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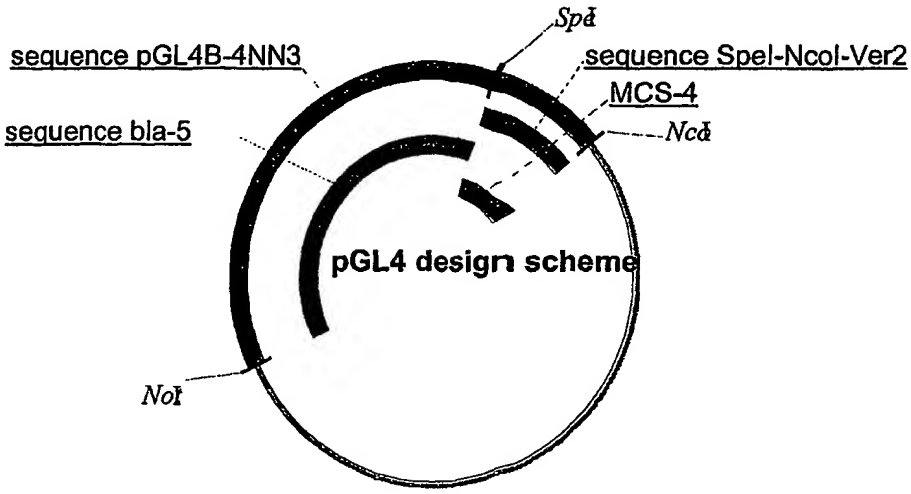

(57) Abstract: A method to prepare synthetic nucleic acid molecules having reduced inappropriate or unintended transcriptional characteristics when expressed in a particular host cell.

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SYNTHETIC NUCLEIC ACID MOLECULE AND METHODS OF PREPARATION

Background

Transcription, the synthesis of an RNA molecule from a sequence of DNA is the first step in gene expression. Sequences which regulate DNA transcription include promoter sequences, polyadenylation signals, transcription factor binding sites and enhancer elements. A promoter is a DNA sequence capable of specific initiation of transcription and consists of three general regions. The core promoter is the sequence where the RNA polymerase and its cofactors bind to the DNA. Immediately upstream of the core promoter is the proximal promoter which contains several transcription factor binding sites that are responsible for the assembly of an activation complex that in turn recruits the polymerase complex. The distal promoter, located further upstream of the proximal promoter also contains transcription factor binding sites. Transcription termination and polyadenylation, like transcription initiation, are site specific and encoded by defined sequences. Enhancers are regulatory regions, containing multiple transcription factor binding sites, that can significantly increase the level of transcription from a responsive promoter regardless of the enhancer's orientation and distance with respect to the promoter as long as the enhancer and promoter are located within the same DNA molecule. The amount of transcript produced from a gene may also be regulated by a post-transcriptional mechanism, the most important being RNA splicing that removes intervening sequences (introns) from a primary transcript between splice donor and splice acceptor sequences.

Natural selection is the hypothesis that genotype-environment interactions occurring at the phenotypic level lead to differential reproductive success of individuals and therefore to modification of the gene pool of a population. Some properties of nucleic acid molecules that are acted upon by natural selection include codon usage frequency, RNA secondary structure, the efficiency of intron splicing, and interactions with transcription factors or other nucleic acid binding proteins. Because of the degenerate nature of the genetic

code, these properties can be optimized by natural selection without altering the corresponding amino acid sequence.

Under some conditions, it is useful to synthetically alter the natural nucleotide sequence encoding a polypeptide to better adapt the polypeptide for alternative applications. A common example is to alter the codon usage frequency of a gene when it is expressed in a foreign host cell. Although redundancy in the genetic code allows amino acids to be encoded by multiple codons, different organisms favor some codons over others. It has been found that the efficiency of protein translation in a non-native host cell can be substantially increased by adjusting the codon usage frequency but maintaining the same gene product (U.S. Patent Nos. 5,096,825, 5,670,356, and 5,874,304).

However, altering codon usage may, in turn, result in the unintentional introduction into a synthetic nucleic acid molecule of inappropriate transcription regulatory sequences. This may adversely effect transcription, resulting in anomalous expression of the synthetic DNA. Anomalous expression is defined as departure from normal or expected levels of expression. For example, transcription factor binding sites located downstream from a promoter have been demonstrated to effect promoter activity (Michael et al., 1990; Lamb et al., 1998; Johnson et al., 1998; Jones et al., 1997). Additionally, it is not uncommon for an enhancer element to exert activity and result in elevated levels of DNA transcription in the absence of a promoter sequence or for the presence of transcription regulatory sequences to increase the basal levels of gene expression in the absence of a promoter sequence.

Thus, what is needed is a method for making synthetic nucleic acid molecules with altered codon usage without also introducing inappropriate or unintended transcription regulatory sequences for expression in a particular host cell.

Summary of the Invention

The invention provides an isolated nucleic acid molecule (a polynucleotide) comprising a synthetic nucleotide sequence having reduced, for instance, 90% or less, e.g., 80%, 78%, 75%, or 70% or less, nucleic acid sequence identity relative to a parent nucleic acid sequence, e.g., a wild-type

nucleic acid sequence, and having fewer regulatory sequences such as transcription regulatory sequences. In one embodiment, the synthetic nucleotide sequence has fewer regulatory sequences than would result if the sequence differences between the synthetic nucleotide sequence and the parent nucleic acid sequence, e.g., optionally the result of differing codons, were randomly selected. In one embodiment, the synthetic nucleotide sequence encodes a polypeptide that has an amino acid sequence that is at least 85%, 90%, 95%, or 99%, or 100%, identical to the amino acid sequence of a naturally-occurring (native or wild-type) corresponding polypeptide (protein). Thus, it is recognized that some specific amino acid changes may also be desirable to alter a particular phenotypic characteristic of a polypeptide encoded by the synthetic nucleotide sequence. Preferably, the amino acid sequence identity is over at least 100 contiguous amino acid residues. In one embodiment of the invention, the codons in the synthetic nucleotide sequence that differ preferably encode the same amino acids as the corresponding codons in the parent nucleic acid sequence.

Hence, in one embodiment, the invention provides an isolated nucleic acid molecule comprising a synthetic nucleotide sequence having a coding region for a selectable or screenable polypeptide, wherein the synthetic nucleotide sequence has 90%, e.g., 80%, or less nucleic acid sequence identity to a parent nucleic acid sequence encoding a corresponding selectable or screenable polypeptide, and wherein the synthetic nucleotide sequence encodes a selectable or screenable polypeptide with at least 85% amino acid sequence identity to the corresponding selectable or screenable polypeptide encoded by the parent nucleic acid sequence. The decreased nucleotide sequence identity may be a result of different codons in the synthetic nucleotide sequence relative to the codons in the parent nucleic acid sequence. The synthetic nucleotide sequence of the invention has a reduced number of regulatory sequences relative to the parent nucleic acid sequence, for example, relative to the average number of regulatory sequences resulting from random selections of codons or nucleotides at the sequences which differ between the synthetic nucleotide sequence and the parent nucleic acid sequence. In one embodiment, a nucleic acid molecule may include a synthetic nucleotide sequence which together with other sequences encodes a selectable or screenable polypeptide. For instance, a synthetic nucleotide

sequence which forms part of an open reading frame for a selectable or screenable polypeptide may include at least 100, 150, 200, 250, 300 or more nucleotides of the open reading, which nucleotides have reduced nucleic acid sequence identity relative to corresponding sequences in a parent nucleic acid sequence. In one embodiment, the parent nucleic acid sequence is SEQ ID NO:1, SEQ ID NO:6, SEQ ID NO:15 or SEQ ID NO:41, the complement thereof, or a sequence that has 90%, 95% or 99% nucleic acid sequence identity thereto.

In one embodiment, the nucleic acid molecule of the invention comprises sequences which have been optimized for expression in mammalian cells, and more preferably, in human cells (see, e.g., WO 02/16944 which discloses methods to optimize sequences for expression in a cell of interest). For instance, nucleic acid molecules may be optimized for expression in eukaryotic cells by introducing a Kozak sequence and/or one or more introns or decreasing the number of other regulatory sequences, and/or altering codon usage to codons employed more frequently in one or more eukaryotic organisms, e.g., codons employed more frequently in an eukaryotic host cell to be transformed with the nucleic acid molecule.

In one embodiment, the synthetic nucleotide sequence is present in a vector, e.g., a plasmid, and such a vector may include other optimized sequences. In one embodiment, the synthetic nucleotide sequence encodes a polypeptide comprising a selectable polypeptide, which synthetic nucleotide sequence has at least 90% or more nucleic acid sequence identity to an open reading frame in a sequence comprising, for example, SEQ ID NO:5, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:30, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:42, SEQ ID NO:44, SEQ ID NO:70, SEQ ID NO:71, SEQ ID NO:72, SEQ ID NO:73, SEQ ID NO:74, SEQ ID NO:80, SEQ ID NO:81, SEQ ID NO:82, SEQ ID NO:83, SEQ ID NO:84, the complement thereof, or a fragment thereof that encodes a polypeptide with substantially the same activity as the corresponding full-length and optionally wild-type (functional) polypeptide, e.g., a polypeptide encoded by SEQ ID NO:1, SEQ ID NO:6, SEQ ID NO:15 or SEQ ID NO:41, or a portion thereof which together with other parent or wild-type sequences encodes a polypeptide with substantially the same activity as the

corresponding full-length and optionally wild-type polypeptide. As used herein, "substantially the same activity" is at least about 70%, e.g., 80%, 90% or more, the activity of a corresponding full-length and optionally wild-type (functional) polypeptide. In one embodiment, an isolated nucleic acid molecule encodes a fusion polypeptide comprising a selectable polypeptide.

Also provided is an isolated nucleic acid molecule comprising a synthetic nucleotide sequence having a coding region for a firefly luciferase, wherein the nucleic acid sequence identity of the synthetic nucleic acid molecule is 90% or less, e.g., 80%, 78%, 75% or less, compared to a parent nucleic acid sequence encoding a firefly luciferase, e.g., a parent nucleic acid sequence having SEQ ID NO:14 or SEQ ID NO:43, which synthetic nucleotide sequence has fewer regulatory sequences including transcription regulatory sequences than would result if the sequence differences, e.g., differing codons, were randomly selected. Preferably, the synthetic nucleotide sequence encodes a polypeptide that has an amino acid sequence that is at least 85%, preferably 90%, and most preferably 95% or 99% identical to the amino acid sequence of a naturally-occurring or parent polypeptide. Thus, it is recognized that some specific amino acid changes may be desirable to alter a particular phenotypic characteristic of the luciferase encoded by the synthetic nucleotide sequence. Preferably, the amino acid sequence identity is over at least 100 contiguous amino acid residues. In one embodiment, the synthetic nucleotide sequence encodes a polypeptide comprising a firefly luciferase, which synthetic nucleotide sequence has at least 90% or more nucleic acid sequence identity to an open reading frame in a sequence comprising, for example, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, the complement thereof, or a fragment thereof that encodes a polypeptide with substantially the same activity as the corresponding full-length and optionally wild-type (functional) polypeptide, e.g., a polypeptide encoded by SEQ ID NO:14 or SEQ ID NO:43, or a portion thereof which together with other sequences encodes a firefly luciferase. For instance, a synthetic nucleotide sequence which forms part of an open reading frame for a firefly luciferase may include at least 100, 150, 200, 250, 300 or more nucleotides of the open reading, which nucleotides have reduced nucleic acid sequence identity relative to corresponding sequences in a parent nucleic acid sequence.

In another embodiment, the invention provides an isolated nucleic acid molecule comprising a synthetic nucleotide sequence which does not include an open reading frame encoding a peptide or polypeptide of interest, e.g., the synthetic nucleotide sequence may have an open reading frame but it does not include sequences that encode a functional or desirable peptide or polypeptide, but may include one or more stop codons in one or more reading frames, one or more poly(A) adenylation sites, and/or a contiguous sequence for two or more restriction endonucleases (restriction enzymes), i.e., a multiple cloning region (also referred to as a multiple cloning site, "MCS"), and which is generally at least 20, e.g., at least 30, nucleotides in length and up to 1000 or more nucleotides, e.g., up to 10,000 nucleotides, which synthetic nucleotide sequence has fewer regulatory sequences such as transcription regulatory sequences relative to a corresponding parent nucleic acid sequence. In one embodiment, the synthetic nucleotide sequence which does not encode a peptide or polypeptide has 90% or less, e.g., 80%, or less nucleic acid sequence identity to a parent nucleic acid sequence, wherein the decreased sequence identity is a result of a reduced number of regulatory sequences in the synthetic nucleotide sequence relative to the parent nucleic acid sequence.

The regulatory sequences which are reduced in the synthetic nucleotide sequence include, but are not limited to, any combination of transcription factor binding sequences, intron splice sites, poly(A) adenylation sites (poly(A) sequences or poly(A) sites hereinafter), enhancer sequences, promoter modules, and/or promoter sequences, e.g., prokaryotic promoter sequences. Generally, a synthetic nucleic acid molecule lacks at least 10%, 20%, 50% or more of the regulatory sequences, for instance lacks substantially all of the regulatory sequences, e.g., 80%, 90% or more, for instance, 95% or more, of the regulatory sequences, present in a corresponding parent or wild-type nucleotide sequence. Regulatory sequences, e.g., transcription regulatory sequences, are well known in the art. The synthetic nucleotide sequence may also have a reduced number of restriction enzyme recognition sites, and may be modified to include selected sequences, e.g., sequences at or near the 5′ and/or 3′ ends of the synthetic nucleotide sequence such as Kozak sequences and/or desirable restriction enzyme recognition sites, for instance, restriction enzyme recognition sites useful

to introduce a synthetic nucleotide sequence to a specified location, e.g., in a multiple cloning region 5' and/or 3' to a nucleic acid sequence of interest.

In one embodiment, the synthetic nucleotide sequence of the invention has a codon composition that differs from that of the parent or wild-type nucleic acid sequence. Preferred codons for use in the invention are those which are employed more frequently than at least one other codon for the same amino acid in a particular organism and/or those that are not low-usage codons in that organism and/or those that are not low-usage codons in the organism used to clone or screen for the expression of the synthetic nucleotide sequence (for example, *E. coli*). Moreover, codons for certain amino acids (i.e., those amino acids that have three or more codons), may include two or more codons that are employed more frequently than the other (non-preferred) codon(s). The presence of codons in a synthetic nucleotide sequence that are employed more frequently in one organism than in another organism results in a synthetic nucleotide sequence which, when introduced into the cells of the organism that employs those codons more frequently, has a reduced risk of aberrant expression and/or is expressed in those cells at a level that may be greater than the expression of the wild type (unmodified) nucleic acid sequence in those cells under some conditions. For example, a synthetic nucleic acid molecule of the invention which encodes a selectable or screenable polypeptide may be expressed at a level that is greater, e.g., at least about 2, 3, 4, 5, 10-fold or more relative to that of the parent or wild-type (unmodified) nucleic acid sequence in a cell or cell extract under identical conditions (such as cell culture conditions, vector backbone, and the like). In one embodiment, the synthetic nucleotide sequence of the invention has a codon composition that differs from that of the parent or wild-type nucleic acid sequence at more than 10%, 20% or more, e.g., 30%, 35%, 40% or more than 45%, e.g., 50%, 55%, 60% or more of the codons.

In one embodiment of the invention, the codons that are different are those employed more frequently in a mammal, while in another embodiment the codons that are different are those employed more frequently in a plant. A particular type of mammal, e.g., human, may have a different set of preferred codons than another type of mammal. Likewise, a particular type of plant may have a different set of preferred codons than another type of plant. In one

embodiment of the invention, the majority of the codons which differ are ones that are preferred codons in a desired host cell and/or are not low usage codons in a particular host cell. Preferred codons for mammals (e.g., humans) and plants are known to the art (e.g., Wada et al., 1990). For example, preferred human codons include, but are not limited to, CGC (Arg), CTG (Leu), AGC (Ser), ACC (Thr), CCC (Pro), GCC (Ala), GGC (Gly), GTG (Val), ACT (Ile), AAG (Lys), AAC (Asn), CAG (Gln), CAC (His), GAG (Glu), GAC (Asp), TAC (Tyr), TGC (Cys) and TTC (Phe) (Wada et al., 1990). Thus, synthetic nucleotide sequences of the invention have a codon composition which differs from a wild type nucleic acid sequence by having an increased number of preferred human codons, e.g. CGC, CTG, TCT, AGC, ACC, CCC, GCC, GGC, GTG, ACT, AAG, AAC, CAG, CAC, GAG, GAC, TAC, TGC, TTC, or any combination thereof. For example, the synthetic nucleotide sequence of the invention may have an increased number of AGC serine-encoding codons, CCC proline-encoding codons, and/or ACC threonine-encoding codons, or any combination thereof, relative to the parent or wild-type nucleic acid sequence. Similarly, synthetic nucleotide sequences having an increased number of codons that are employed more frequently in plants, have a codon composition which differs from a wild-type nucleic acid sequence by having an increased number of the plant codons including, but not limited to, CGC (Arg), CTT (Leu), TCT (Ser), TCC (Ser), ACC (Thr), CCA (Pro), CCT (Pro), GCT (Ser), GGA (Gly), GTG (Val), ATC (Ile), ATT (Ile), AAG (Lys), AAC (Asn), CAA (Gln), CAC (His), GAG (Glu), GAC (Asp), TAC (Tyr), TGC (Cys), TTC (Phe), or any combination thereof (Murray et al., 1989). Preferred codons may differ for different types of plants (Wada et al., 1990).

The nucleotide substitutions in the synthetic nucleic acid sequence may be influenced by many factors such as, for example, the desire to have an increased number of nucleotide substitutions such as those resulting in a silent nucleotide substitution (encodes the same amino acid) and/or decreased number of regulatory sequences. Under some circumstances (e.g., to permit removal of a transcription factor binding site) it may be desirable to replace a non-preferred codon with a codon other than a preferred codon or a codon other than the preferred codon in order to decrease the number of regulatory sequences.

The invention also provides an expression cassette or vector. The expression cassette or vector of the invention comprises a synthetic nucleotide sequence of the invention operatively linked to a promoter that is functional in a cell or comprises a synthetic nucleotide sequence, respectively. Preferred promoters are those functional in mammalian cells and those functional in plant cells. Optionally, the expression cassette may include other sequences, e.g., one or more restriction enzyme recognition sequences 5′ and/or 3′ to an open reading frame for a selectable polypeptide or luciferase and/or a Kozak sequence, and be a part of a larger polynucleotide molecule such as a plasmid, cosmid, artificial chromosome or vector, e.g., a viral vector, which may include a multiple cloning region for other sequences, e.g., promoters, enhancers, other open reading frames and/or poly(A) sites. In one embodiment, a vector of the invention includes SEQ ID NO:88, SEQ ID NO:89, SEQ ID NO:90, the complement thereof, or a sequence which has at least 80% nucleic acid sequence identity thereto and encodes a selectable and/or screenable polypeptide.

In one embodiment, the synthetic nucleotide sequence encoding a selectable or screenable polypeptide is introduced into a vector backbone, e.g., one which optionally has a poly(A) site 3′ to the synthetic nucleotide sequence, a gene useful for selecting transformed prokaryotic cells which optionally is a synthetic sequence, a gene useful for selecting transformed eukaryotic cells which optionally is a synthetic sequence, a noncoding region for decreasing transcription and/or translation into adjacent linked desirable open reading frames, and/or a multiple cloning region 5′ and/or 3′ to the synthetic nucleotide sequence encoding a selectable or screenable polypeptide which optionally includes one or more protein destabilization sequences (see U.S. application Serial No. 10/664,341, filed September 16, 2003, the disclosure of which is incorporated by reference herein). In one embodiment, the vector having a synthetic nucleotide sequence encoding a selectable or screenable polypeptide may lack a promoter and/or enhancer which is operably linked to that synthetic sequence. In another embodiment, the invention provides a vector comprising a promoter, e.g., a prokaryotic or eukaryotic promoter, operably linked to a synthetic nucleotide sequence encoding a selectable or screenable polypeptide. Such vectors optionally include one or more multiple cloning regions, such as

ones that are useful to introduce an additional open reading frame and/or a promoter for expression of the open reading frame which promoter optionally is different than the promoter for the selectable or screenable polypeptide, and/or a prokaryotic origin of replication. A "vector backbone" as used herein may include sequences (open reading frames) useful to identify cells with those sequences, e.g., in prokaryotic cells, their promoters, an origin of replication for vector maintenance, e.g., in prokaryotic cells, and optionally one or more other sequences including multiple cloning regions e.g., for insertion of a promoter and/or open reading frame of interest, and sequences which inhibit transcription and/or translation.

Also provided is a host cell comprising the synthetic nucleotide sequence of the invention, an isolated polypeptide (e.g., a fusion polypeptide encoded by the synthetic nucleotide sequence of the invention), and compositions and kits comprising the synthetic nucleotide sequence of the invention, a polypeptide encoded thereby, or an expression cassette or vector comprising the synthetic nucleotide sequence in suitable container means and, optionally, instruction means. The host cell may be an eukaryotic cell such as a plant or vertebrate cell, e.g., a mammalian cell, including but not limited to a human, non-human primate, canine, feline, bovine, equine, ovine or rodent (e.g., rabbit, rat, ferret, hamster, or mouse) cell or a prokaryotic cell.

The invention also provides a method to prepare a synthetic nucleotide sequence of the invention by genetically altering a parent, e.g., a wild-type or synthetic, nucleic acid sequence. The method comprises altering (e.g., decreasing or eliminating) a plurality of regulatory sequences in a parent nucleic acid sequence, e.g., one which encodes a selectable or screenable polypeptide or one which does not encode a peptide or polypeptide, to yield a synthetic nucleotide sequence which has a decreased number of regulatory sequences and, if the synthetic nucleotide sequence encodes a polypeptide, it preferably encodes the same amino acids as the parent nucleic acid molecule. The transcription regulatory sequences which are reduced include but are not limited to any of transcription factor binding sequences, intron splice sites, poly(A) sites, enhancer sequences, promoter modules, and/or promoter sequences. Preferably, the alteration of sequences in the synthetic nucleotide sequence does not result in an

increase in regulatory sequences. In one embodiment, the synthetic nucleotide sequence encodes a polypeptide that has at least 85%, 90%, 95% or 99%, or 100%, contiguous amino acid sequence identity to the amino acid sequence of the polypeptide encoded by the parent nucleic acid sequence.

Thus, in one embodiment, a method to prepare a synthetic nucleic acid molecule comprising an open reading frame is provided. The method includes altering the codons and/or regulatory sequences in a parent nucleic acid sequence which encodes a reporter protein such, as a firefly luciferase or a selectable polypeptide such as one encoding resistance to ampicillin, puromycin, hygromycin or neomycin, to yield a synthetic nucleotide sequence which encodes a corresponding reporter polypeptide and which has for instance at least 10% or more, e.g., 20%, 30%, 40%, 50% or more, fewer regulatory sequences relative to the parent nucleic acid sequence. The synthetic nucleotide sequence has 90%, e.g., 85%, 80%, or 78%, or less nucleic acid sequence identity to the parent nucleic acid sequence and encodes a polypeptide with at least 85% amino acid sequence identity to the polypeptide encoded by the parent nucleic acid sequence. The regulatory sequences which are altered include transcription factor binding sequences, intron splice sites, poly(A) sites, promoter modules, and/or promoter sequences. In one embodiment, the synthetic nucleic acid sequence hybridizes under medium stringency hybridization but not stringent conditions to the parent nucleic acid sequence or the complement thereof. In one embodiment, the codons which differ encode the same amino acids as the corresponding codons in the parent nucleic acid sequence.

Also provided is a synthetic (including a further synthetic) nucleotide sequence prepared by the methods of the invention, e.g., a further synthetic nucleotide sequence in which introduced regulatory sequences or restriction endonuclease recognition sequences are optionally removed. Thus, the method of the invention may be employed to alter the codon usage frequency and/or decrease the number of regulatory sequences in any open reading frame or to decrease the number of regulatory sequences in any nucleic acid sequence, e.g., a noncoding sequence. Preferably, the codon usage frequency in a synthetic nucleotide sequence which encodes a selectable or screenable polypeptide is altered to reflect that of the host organism desired for expression of that

nucleotide sequence while also decreasing the number of potential regulatory sequences relative to the parent nucleic acid molecule.

Also provided is a method to prepare a synthetic nucleic acid molecule which does not code for a peptide or polypeptide. The method includes altering the nucleotides in a parent nucleic acid sequence having at least 20 nucleotides which optionally does not code for a functional or desirable peptide or polypeptide and which optionally may include sequences which inhibit transcription and/or translation, to yield a synthetic nucleotide sequence which does not include an open reading frame encoding a peptide or polypeptide of interest, e.g., the synthetic nucleotide sequence may have an open reading frame but it does not include sequences that encode a functional or desirable peptide or polypeptide, but may include one or more stop codons in one or more reading frames, one or more poly(A) adenylation sites, and/or a contiguous sequence for two or more restriction endonucleases, i.e., a multiple cloning region. The synthetic nucleotide sequence is generally at least 20, e.g., at least 30, nucleotides in length and up to 1000 or more nucleotides, e.g., up to 10,000 nucleotides, and has fewer regulatory sequences such as transcription regulatory sequences relative to a corresponding parent nucleic acid sequence which does not code for a peptide or polypeptide, e.g., a parent nucleic acid sequence which optionally includes sequences which inhibit transcription and/or translation. The nucleotides are altered to reduce one or more regulatory sequences, e.g., transcription factor binding sequences, intron splice sites, poly(A) sites, enhancer sequences, promoter modules, and/or promoter sequences, in the parent nucleic acid sequence.

The invention also provides a method to prepare an expression vector. The method includes providing a linearized plasmid having a nucleic molecule including a synthetic nucleotide sequence of the invention which encodes a selectable or screenable polypeptide which is flanked at the 5′ and/or 3′ end by a multiple cloning region. The plasmid is linearized by contacting the plasmid with at least one restriction endonuclease which cleaves in the multiple cloning region. The linearized plasmid and an expression cassette having ends compatible with the ends in the linearized plasmid are annealed, yielding an expression vector. In one embodiment, the plasmid is linearized by cleavage by

at least two restriction endonucleases, only one of which cleaves in the multiple cloning region.

Also provided is a method to clone a promoter or open reading frame. The method includes comprising providing a linearized plasmid having a multiple cloning region and a synthetic sequence of the invention which encodes a selectable or screenable polypeptide and/or a synthetic sequence of the invention which does not encode a peptide or polypeptide, which is plasmid is linearized by contacting the plasmid with at least two restriction endonucleases at least one of which cleaves in the multiple cloning region; and annealing the linearized plasmid with DNA having a promoter or an open reading frame with ends compatible with the ends of the linearized plasmid.

Exemplary methods to prepare synthetic sequences for firefly luciferase and a number of selectable polypeptide nucleic acid sequences, as well as non-coding regions present in a vector backbone, are described hereinbelow. For instance, the methods may produce synthetic selectable polypeptide nucleic acid molecules which exhibit similar or significantly enhanced levels of mammalian expression without negatively effecting other desirable physical or biochemical properties and which were also largely devoid of regulatory elements.

Clearly, the present invention has applications with many genes and across many fields of science including, but not limited to, life science research, agrigenetics, genetic therapy, developmental science and pharmaceutical development.

Brief Description of the Figures

Figure 1. Codons and their corresponding amino acids.

Figure 2. Design scheme for the pGL4 vector.

Detailed Description of the Invention

Definitions

The term "nucleic acid molecule" or "nucleic acid sequence" as used herein, refers to nucleic acid, DNA or RNA, that comprises noncoding or coding sequences. Coding sequences are necessary for the production of a polypeptide or protein precursor. The polypeptide can be encoded by a full-length coding

sequence or by any portion of the coding sequence, as long as the desired protein activity is retained. Noncoding sequences refer to nucleic acids which do not code for a polypeptide or protein precursor, and may include regulatory elements such as transcription factor binding sites, poly(A) sites, restriction endonuclease sites, stop codons and/or promoter sequences.

A "synthetic" nucleic acid sequence is one which is not found in nature, i.e., it has been derived using molecular biological, chemical and/or informatic techniques.

A "nucleic acid", as used herein, is a covalently linked sequence of nucleotides in which the 3′ position of the pentose of one nucleotide is joined by a phosphodiester group to the 5′ position of the pentose of the next, and in which the nucleotide residues (bases) are linked in specific sequence, i.e., a linear order of nucleotides. A "polynucleotide", as used herein, is a nucleic acid containing a sequence that is greater than about 100 nucleotides in length. An "oligonucleotide" or "primer", as used herein, is a short polynucleotide or a portion of a polynucleotide. An oligonucleotide typically contains a sequence of about two to about one hundred bases. The word "oligo" is sometimes used in place of the word "oligonucleotide".

Nucleic acid molecules are said to have a "5′-terminus" (5′ end) and a "3′-terminus" (3′ end) because nucleic acid phosphodiester linkages occur to the 5′ carbon and 3′ carbon of the pentose ring of the substituent mononucleotides. The end of a polynucleotide at which a new linkage would be to a 5′ carbon is its 5′ terminal nucleotide. The end of a polynucleotide at which a new linkage would be to a 3′ carbon is its 3′ terminal nucleotide. A terminal nucleotide, as used herein, is the nucleotide at the end position of the 3′- or 5′-terminus.

DNA molecules are said to have "5′ ends" and "3′ ends" because mononucleotides are reacted to make oligonucleotides in a manner such that the 5′ phosphate of one mononucleotide pentose ring is attached to the 3′ oxygen of its neighbor in one direction via a phosphodiester linkage. Therefore, an end of an oligonucleotides referred to as the "5′ end" if its 5′ phosphate is not linked to the 3′ oxygen of a mononucleotide pentose ring and as the "3′ end" if its 3′ oxygen is not linked to a 5′ phosphate of a subsequent mononucleotide pentose

ring.

As used herein, a nucleic acid sequence, even if internal to a larger oligonucleotide or polynucleotide, also may be said to have 5′ and 3′ ends. In either a linear or circular DNA molecule, discrete elements are referred to as being "upstream" or 5′ of the "downstream" or 3′ elements. This terminology reflects the fact that transcription proceeds in a 5′ to 3′ fashion along the DNA strand. Typically, promoter and enhancer elements that direct transcription of a linked gene (e.g., open reading frame or coding region) are generally located 5′ or upstream of the coding region. However, enhancer elements can exert their effect even when located 3′ of the promoter element and the coding region. Transcription termination and polyadenylation signals are located 3′ or downstream of the coding region.

The term "codon" as used herein, is a basic genetic coding unit, consisting of a sequence of three nucleotides that specify a particular amino acid to be incorporation into a polypeptide chain, or a start or stop signal. The term "coding region" when used in reference to structural genes refers to the nucleotide sequences that encode the amino acids found in the nascent polypeptide as a result of translation of a mRNA molecule. Typically, the coding region is bounded on the 5′ side by the nucleotide triplet "ATG" which encodes the initiator methionine and on the 3′ side by a stop codon (e.g., TAA, TAG, TGA). In some cases the coding region is also known to initiate by a nucleotide triplet "TTG".

By "protein", "polypeptide" or "peptide" is meant any chain of amino acids, regardless of length or post-translational modification (e.g., glycosylation or phosphorylation). The nucleic acid molecules of the invention may also encode a variant of a naturally-occurring protein or a fragment thereof. Preferably, such a variant protein has an amino acid sequence that is at least 85%, preferably 90%, and most preferably 95% or 99% identical to the amino acid sequence of the naturally-occurring (native or wild-type) protein from which it is derived.

Polypeptide molecules are said to have an "amino terminus" (N-terminus) and a "carboxy terminus" (C-terminus) because peptide linkages occur between the backbone amino group of a first amino acid residue and the backbone

carboxyl group of a second amino acid residue. The terms "N-terminal" and "C-terminal" in reference to polypeptide sequences refer to regions of polypeptides including portions of the N-terminal and C-terminal regions of the polypeptide, respectively. A sequence that includes a portion of the N-terminal region of a polypeptide includes amino acids predominantly from the N-terminal half of the polypeptide chain, but is not limited to such sequences. For example, an N-terminal sequence may include an interior portion of the polypeptide sequence including bases from both the N-terminal and C-terminal halves of the polypeptide. The same applies to C-terminal regions. N-terminal and C-terminal regions may, but need not, include the amino acid defining the ultimate N-terminus and C-terminus of the polypeptide, respectively.

The term "wild-type" as used herein, refers to a gene or gene product that has the characteristics of that gene or gene product isolated from a naturally occurring source. A wild-type gene is that which is most frequently observed in a population and is thus arbitrarily designated the "wild-type" form of the gene. In contrast, the term "mutant" refers to a gene or gene product that displays modifications in sequence and/or functional properties (i.e., altered characteristics) when compared to the wild-type gene or gene product. It is noted that naturally-occurring mutants can be isolated; these are identified by the fact that they have altered characteristics when compared to the wild-type gene or gene product.

The term "recombinant protein" or "recombinant polypeptide" as used herein refers to a protein molecule expressed from a recombinant DNA molecule. In contrast, the term "native protein" is used herein to indicate a protein isolated from a naturally occurring (i.e., a nonrecombinant) source. Molecular biological techniques may be used to produce a recombinant form of a protein with identical properties as compared to the native form of the protein.

The term "fusion polypeptide" refers to a chimeric protein containing a protein of interest (e.g., luciferase) joined to a heterologous sequence (e.g., a non-luciferase amino acid or protein).

The terms "cell," "cell line," "host cell," as used herein, are used interchangeably, and all such designations include progeny or potential progeny of these designations. By "transformed cell" is meant a cell into which (or into

an ancestor of which) has been introduced a nucleic acid molecule of the invention, e.g., via transient transfection. Optionally, a nucleic acid molecule synthetic gene of the invention may be introduced into a suitable cell line so as to create a stably-transfected cell line capable of producing the protein or polypeptide encoded by the synthetic gene. Vectors, cells, and methods for constructing such cell lines are well known in the art. The words "transformants" or "transformed cells" include the primary transformed cells derived from the originally transformed cell without regard to the number of transfers. All progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations. Nonetheless, mutant progeny that have the same functionality as screened for in the originally transformed cell are included in the definition of transformants.

Nucleic acids are known to contain different types of mutations. A "point" mutation refers to an alteration in the sequence of a nucleotide at a single base position from the wild type sequence. Mutations may also refer to insertion or deletion of one or more bases, so that the nucleic acid sequence differs from the wild-type sequence.

The term "homology" refers to a degree of complementarity between two or more sequences. There may be partial homology or complete homology (i.e., identity). Homology is often measured using sequence analysis software (e.g., EMBOSS, the European Molecular Biology Open Software Suite available at http://www.hgmp.mrc.ac.uk/Software/EMBOSS/overview/html). Such software matches similar sequences by assigning degrees of homology to various substitutions, deletions, insertions, and other modifications. Conservative substitutions typically include substitutions within the following groups: glycine, alanine; valine, isoleucine, leucine; aspartic acid, glutamic acid, asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine.

The term "isolated" when used in relation to a nucleic acid, as in "isolated oligonucleotide" or "isolated polynucleotide" refers to a nucleic acid sequence that is identified and separated from at least one contaminant with which it is ordinarily associated in its source. Thus, an isolated nucleic acid is present in a form or setting that is different from that in which it is found in nature. In

contrast, non-isolated nucleic acids (e.g., DNA and RNA) are found in the state they exist in nature. For example, a given DNA sequence (e.g., a gene) is found on the host cell chromosome in proximity to neighboring genes; RNA sequences (e.g., a specific mRNA sequence encoding a specific protein), are found in the cell as a mixture with numerous other mRNAs that encode a multitude of proteins. However, isolated nucleic acid includes, by way of example, such nucleic acid in cells ordinarily expressing that nucleic acid where the nucleic acid is in a chromosomal location different from that of natural cells, or is otherwise flanked by a different nucleic acid sequence than that found in nature. The isolated nucleic acid or oligonucleotide may be present in single-stranded or double-stranded form. When an isolated nucleic acid or oligonucleotide is to be utilized to express a protein, the oligonucleotide contains at a minimum, the sense or coding strand (i.e., the oligonucleotide may be single-stranded), but may contain both the sense and anti-sense strands (i.e., the oligonucleotide may be double-stranded).

The term "isolated" when used in relation to a polypeptide, as in "isolated protein" or "isolated polypeptide" refers to a polypeptide that is identified and separated from at least one contaminant with which it is ordinarily associated in its source. Thus, an isolated polypeptide is present in a form or setting that is different from that in which it is found in nature. In contrast, non-isolated polypeptides (e.g., proteins and enzymes) are found in the state they exist in nature.

The term "purified" or "to purify" means the result of any process that removes some of a contaminant from the component of interest, such as a protein or nucleic acid. The percent of a purified component is thereby increased in the sample.

The term "operably linked" as used herein refer to the linkage of nucleic acid sequences in such a manner that a nucleic acid molecule capable of directing the transcription of a given gene and/or the synthesis of a desired protein molecule is produced. The term also refers to the linkage of sequences encoding amino acids in such a manner that a functional (e.g., enzymatically active, capable of binding to a binding partner, capable of inhibiting, etc.) protein or polypeptide is produced.

The term "recombinant DNA molecule" means a hybrid DNA sequence comprising at least two nucleotide sequences not normally found together in nature.

The term "vector" is used in reference to nucleic acid molecules into which fragments of DNA may be inserted or cloned and can be used to transfer DNA segment(s) into a cell and capable of replication in a cell. Vectors may be derived from plasmids, bacteriophages, viruses, cosmids, and the like.

The terms "recombinant vector" and "expression vector" as used herein refer to DNA or RNA sequences containing a desired coding sequence and appropriate DNA or RNA sequences necessary for the expression of the operably linked coding sequence in a particular host organism. Prokaryotic expression vectors include a promoter, a ribosome binding site, an origin of replication for autonomous replication in a host cell and possibly other sequences, e.g. an optional operator sequence, optional restriction enzyme sites. A promoter is defined as a DNA sequence that directs RNA polymerase to bind to DNA and to initiate RNA synthesis. Eukaryotic expression vectors include a promoter, optionally a polyadenlyation signal and optionally an enhancer sequence.

A polynucleotide having a nucleotide sequence encoding a protein or polypeptide means a nucleic acid sequence comprising the coding region of a gene, or in other words the nucleic acid sequence encodes a gene product. The coding region may be present in either a cDNA, genomic DNA or RNA form. When present in a DNA form, the oligonucleotide may be single-stranded (i.e., the sense strand) or double-stranded. Suitable control elements such as enhancers/promoters, splice junctions, polyadenylation signals, etc. may be placed in close proximity to the coding region of the gene if needed to permit proper initiation of transcription and/or correct processing of the primary RNA transcript. Alternatively, the coding region utilized in the expression vectors of the present invention may contain endogenous enhancers/promoters, splice junctions, intervening sequences, polyadenylation signals, etc. In further embodiments, the coding region may contain a combination of both endogenous and exogenous control elements.

The term "regulatory element" or "regulatory sequence" refers to a genetic element or sequence that controls some aspect of the expression of

nucleic acid sequence(s). For example, a promoter is a regulatory element that facilitates the initiation of transcription of an operably linked coding region. Other regulatory elements include, but are not limited to, transcription factor binding sites, splicing signals, polyadenylation signals, termination signals and enhancer elements.

Transcriptional control signals in eukaryotes comprise "promoter" and "enhancer" elements. Promoters and enhancers consist of short arrays of DNA sequences that interact specifically with cellular proteins involved in transcription. Promoter and enhancer elements have been isolated from a variety of eukaryotic sources including genes in yeast, insect and mammalian cells. Promoter and enhancer elements have also been isolated from viruses and analogous control elements, such as promoters, are also found in prokaryotes. The selection of a particular promoter and enhancer depends on the cell type used to express the protein of interest. Some eukaryotic promoters and enhancers have a broad host range while others are functional in a limited subset of cell types. For example, the SV40 early gene enhancer is very active in a wide variety of cell types from many mammalian species and has been widely used for the expression of proteins in mammalian cells. Two other examples of promoter/enhancer elements active in a broad range of mammalian cell types are those from the human elongation factor 1 gene (Uetsuki et al., 1989; Kim et al., 1990; and Mizushima and Nagata, 1990) and the long terminal repeats of the Rous sarcoma virus (Gorman et al., 1982); and the human cytomegalovirus (Boshart et al., 1985).

The term "promoter/enhancer" denotes a segment of DNA containing sequences capable of providing both promoter and enhancer functions (i.e., the functions provided by a promoter element and an enhancer element as described above). For example, the long terminal repeats of retroviruses contain both promoter and enhancer functions. The enhancer/promoter may be "endogenous" or "exogenous" or "heterologous." An "endogenous" enhancer/promoter is one that is naturally linked with a given gene in the genome. An "exogenous" or "heterologous" enhancer/promoter is one that is placed in juxtaposition to a gene by means of genetic manipulation (i.e., molecular biological techniques) such that transcription of the gene is directed by the linked enhancer/promoter.

The presence of "splicing signals" on an expression vector often results in higher levels of expression of the recombinant transcript in eukaryotic host cells. Splicing signals mediate the removal of introns from the primary RNA transcript and consist of a splice donor and acceptor site (Sambrook et al., 1989). A commonly used splice donor and acceptor site is the splice junction from the 16S RNA of SV40.

Efficient expression of recombinant DNA sequences in eukaryotic cells requires expression of signals directing the efficient termination and polyadenylation of the resulting transcript. Transcription termination signals are generally found downstream of the polyadenylation signal and are a few hundred nucleotides in length. The term "poly(A) site" or "poly(A) sequence" as used herein denotes a DNA sequence which directs both the termination and polyadenylation of the nascent RNA transcript. Efficient polyadenylation of the recombinant transcript is desirable, as transcripts lacking a poly(A) tail are unstable and are rapidly degraded. The poly(A) signal utilized in an expression vector may be "heterologous" or "endogenous." An endogenous poly(A) signal is one that is found naturally at the 3′ end of the coding region of a given gene in the genome. A heterologous poly(A) signal is one which has been isolated from one gene and positioned 3′ to another gene. A commonly used heterologous poly(A) signal is the SV40 poly(A) signal. The SV40 poly(A) signal is contained on a 237 bp *Bam*H I/*Bcl* I restriction fragment and directs both termination and polyadenylation (Sambrook et al., 1989).

Eukaryotic expression vectors may also contain "viral replicons "or "viral origins of replication." Viral replicons are viral DNA sequences which allow for the extrachromosomal replication of a vector in a host cell expressing the appropriate replication factors. Vectors containing either the SV40 or polyoma virus origin of replication replicate to high copy number (up to 10^4 copies/cell) in cells that express the appropriate viral T antigen. In contrast, vectors containing the replicons from bovine papillomavirus or Epstein-Barr virus replicate extrachromosomally at low copy number (about 100 copies/cell).

The term "*in vitro*" refers to an artificial environment and to processes or reactions that occur within an artificial environment. *In vitro* environments include, but are not limited to, test tubes and cell lysates. The term "*in vivo*"

refers to the natural environment (e.g., an animal or a cell) and to processes or reactions that occur within a natural environment.

The term "expression system" refers to any assay or system for determining (e.g., detecting) the expression of a gene of interest. Those skilled in the field of molecular biology will understand that any of a wide variety of expression systems may be used. A wide range of suitable mammalian cells are available from a wide range of sources (e.g., the American Type Culture Collection, Rockland, MD). The method of transformation or transfection and the choice of expression vehicle will depend on the host system selected. Transformation and transfection methods are described, e.g., in Ausubel et al., 1992. Expression systems include *in vitro* gene expression assays where a gene of interest (e.g., a reporter gene) is linked to a regulatory sequence and the expression of the gene is monitored following treatment with an agent that inhibits or induces expression of the gene. Detection of gene expression can be through any suitable means including, but not limited to, detection of expressed mRNA or protein (e.g., a detectable product of a reporter gene) or through a detectable change in the phenotype of a cell expressing the gene of interest. Expression systems may also comprise assays where a cleavage event or other nucleic acid or cellular change is detected.

All amino acid residues identified herein are in the natural L-configuration. In keeping with standard polypeptide nomenclature, abbreviations for amino acid residues are as shown in the following Table of Correspondence.

TABLE OF CORRESPONDENCE

1-Letter	3-Letter	AMINO ACID
Y	Tyr	L-tyrosine
G	Gly	L-glycine
F	Phe	L-phenylalanine
M	Met	L-methionine
A	Ala	L-alanine
S	Ser	L-serine
I	Ile	L-isoleucine

L	Leu	L-leucine
T	Thr	L-threonine
V	Val	L-valine
P	Pro	L-proline
K	Lys	L-lysine
H	His	L-histidine
Q	Gln	L-glutamine
E	Glu	L-glutamic acid
W	Trp	L-tryptophan
R	Arg	L-arginine
D	Asp	L-aspartic acid
N	Asn	L-asparagine
C	Cys	L-cysteine

The terms "complementary" or "complementarity" are used in reference to a sequence of nucleotides related by the base-pairing rules. For example, for the sequence 5′ "A-G-T" 3′, is complementary to the sequence 3′ "T-C-A" 5′. Complementarity may be "partial," in which only some of the nucleic acids' bases are matched according to the base pairing rules. Or, there may be "complete" or "total" complementarity between the nucleic acids. The degree of complementarity between nucleic acid strands has significant effects on the efficiency and strength of hybridization between nucleic acid strands. This is of particular importance in amplification reactions, as well as detection methods which depend upon hybridization of nucleic acids.

When used in reference to a double-stranded nucleic acid sequence such as a cDNA or a genomic clone, the term "substantially homologous" refers to any probe which can hybridize to either or both strands of the double-stranded nucleic acid sequence under conditions of low stringency as described herein.

"Probe" refers to an oligonucleotide designed to be sufficiently complementary to a sequence in a denatured nucleic acid to be probed (in relation to its length) and is bound under selected stringency conditions.

"Hybridization" and "binding" in the context of probes and denatured nucleic acids are used interchangeably. Probes that are hybridized or bound to denatured nucleic acids are base paired to complementary sequences in the

polynucleotide. Whether or not a particular probe remains base paired with the polynucleotide depends on the degree of complementarity, the length of the probe, and the stringency of the binding conditions. The higher the stringency, the higher must be the degree of complementarity and/or the longer the probe.

The term "hybridization" is used in reference to the pairing of complementary nucleic acid strands. Hybridization and the strength of hybridization (i.e., the strength of the association between nucleic acid strands) is impacted by many factors well known in the art including the degree of complementarity between the nucleic acids, stringency of the conditions involved such as the concentration of salts, the Tm (melting temperature) of the formed hybrid, the presence of other components (e.g., the presence or absence of polyethylene glycol), the molarity of the hybridizing strands and the G:C content of the nucleic acid strands.

The term "stringency" is used in reference to the conditions of temperature, ionic strength, and the presence of other compounds, under which nucleic acid hybridizations are conducted. With "high stringency" conditions, nucleic acid base pairing will occur only between nucleic acid fragments that have a high frequency of complementary base sequences. Thus, conditions of "medium" or "low" stringency are often required when it is desired that nucleic acids that are not completely complementary to one another be hybridized or annealed together. The art knows well that numerous equivalent conditions can be employed to comprise medium or low stringency conditions. The choice of hybridization conditions is generally evident to one skilled in the art and is usually guided by the purpose of the hybridization, the type of hybridization (DNA-DNA or DNA-RNA), and the level of desired relatedness between the sequences (e.g., Sambrook et al., 1989; Nucleic Acid Hybridization, A Practical Approach, IRL Press, Washington D.C., 1985, for a general discussion of the methods).

The stability of nucleic acid duplexes is known to decrease with increasing numbers of mismatched bases, and further to be decreased to a greater or lesser degree depending on the relative positions of mismatches in the hybrid duplexes. Thus, the stringency of hybridization can be used to maximize or minimize stability of such duplexes. Hybridization stringency can be altered by:

adjusting the temperature of hybridization; adjusting the percentage of helix destabilizing agents, such as formamide, in the hybridization mix; and adjusting the temperature and/or salt concentration of the wash solutions. For filter hybridizations, the final stringency of hybridizations often is determined by the salt concentration and/or temperature used for the post-hybridization washes.

"High stringency conditions" when used in reference to nucleic acid hybridization comprise conditions equivalent to binding or hybridization at 42°C in a solution consisting of 5X SSPE (43.8 g/l NaCl, 6.9 g/l NaH_2PO_4 H_2O and 1.85 g/l EDTA, pH adjusted to 7.4 with NaOH), 0.5% SDS, 5X Denhardt's reagent and 100 μg/ml denatured salmon sperm DNA followed by washing in a solution comprising 0.1X SSPE, 1.0% SDS at 42°C when a probe of about 500 nucleotides in length is employed.

"Medium stringency conditions" when used in reference to nucleic acid hybridization comprise conditions equivalent to binding or hybridization at 42°C in a solution consisting of 5X SSPE (43.8 g/l NaCl, 6.9 g/l NaH_2PO_4 H_2O and 1.85 g/l EDTA, pH adjusted to 7.4 with NaOH), 0.5% SDS, 5X Denhardt's reagent and 100 μg/ml denatured salmon sperm DNA followed by washing in a solution comprising 1.0X SSPE, 1.0% SDS at 42°C when a probe of about 500 nucleotides in length is employed.

"Low stringency conditions" comprise conditions equivalent to binding or hybridization at 42°C in a solution consisting of 5X SSPE (43.8 g/l NaCl, 6.9 g/l NaH_2PO_4 H_2O and 1.85 g/l EDTA, pH adjusted to 7.4 with NaOH), 0.1% SDS, 5X Denhardt's reagent [50X Denhardt's contains per 500 ml: 5 g Ficoll (Type 400, Pharmacia), 5 g BSA (Fraction V; Sigma)] and 100 g/ml denatured salmon sperm DNA followed by washing in a solution comprising 5X SSPE, 0.1% SDS at 42°C when a probe of about 500 nucleotides in length is employed.

The term "T_m" is used in reference to the "melting temperature". The melting temperature is the temperature at which 50% of a population of double-stranded nucleic acid molecules becomes dissociated into single strands. The equation for calculating the T_m of nucleic acids is well-known in the art. The Tm of a hybrid nucleic acid is often estimated using a formula adopted from hybridization assays in 1 M salt, and commonly used for calculating Tm for PCR primers: [(number of A + T) x 2°C + (number of G+C) x 4°C]. (C.R. Newton et

al., PCR, 2nd Ed., Springer-Verlag (New York, 1997), p. 24). This formula was found to be inaccurate for primers longer than 20 nucleotides. (Id.) Another simple estimate of the T_m value may be calculated by the equation: $T_m = 81.5 + 0.41(\% G + C)$, when a nucleic acid is in aqueous solution at 1 M NaCl. (e.g., Anderson and Young, Quantitative Filter Hybridization, in Nucleic Acid Hybridization, 1985). Other more sophisticated computations exist in the art which take structural as well as sequence characteristics into account for the calculation of T_m. A calculated T_m is merely an estimate; the optimum temperature is commonly determined empirically.

The term "promoter/enhancer" denotes a segment of DNA containing sequences capable of providing both promoter and enhancer functions (i.e., the functions provided by a promoter element and an enhancer element as described above). For example, the long terminal repeats of retroviruses contain both promoter and enhancer functions. The enhancer/promoter may be "endogenous" or "exogenous" or "heterologous." An "endogenous" enhancer/promoter is one that is naturally linked with a given gene in the genome. An "exogenous" or "heterologous" enhancer/promoter is one that is placed in juxtaposition to a gene by means of genetic manipulation (i.e., molecular biological techniques) such that transcription of the gene is directed by the linked enhancer/promoter.

The term "sequence homology" means the proportion of base matches between two nucleic acid sequences or the proportion of amino acid matches between two amino acid sequences. When sequence homology is expressed as a percentage, e.g., 50%, the percentage denotes the proportion of matches over the length of sequence from one sequence that is compared to some other sequence. Gaps (in either of the two sequences) are permitted to maximize matching; gap lengths of 15 bases or less are usually used, 6 bases or less are preferred with 2 bases or less more preferred. When using oligonucleotides as probes or treatments, the sequence homology between the target nucleic acid and the oligonucleotide sequence is generally not less than 17 target base matches out of 20 possible oligonucleotide base pair matches (85%); preferably not less than 9 matches out of 10 possible base pair matches (90%), and more preferably not less than 19 matches out of 20 possible base pair matches (95%).

Two amino acid sequences are homologous if there is a partial or complete identity between their sequences. For example, 85% homology means that 85% of the amino acids are identical when the two sequences are aligned for maximum matching. Gaps (in either of the two sequences being matched) are allowed in maximizing matching; gap lengths of 5 or less are preferred with 2 or less being more preferred. Alternatively and preferably, two protein sequences (or polypeptide sequences derived from them of at least 100 amino acids in length) are homologous, as this term is used herein, if they have an alignment score of at more than 5 (in standard deviation units) using the program ALIGN with the mutation data matrix and a gap penalty of 6 or greater. See Dayhoff, M. O., in Atlas of Protein Sequence and Structure, 1972, volume 5, National Biomedical Research Foundation, pp. 101-110, and Supplement 2 to this volume, pp. 1-10. The two sequences or parts thereof are more preferably homologous if their amino acids are greater than or equal to 85% identical when optimally aligned using the ALIGN program.

The following terms are used to describe the sequence relationships between two or more polynucleotides: "reference sequence", "comparison window", "sequence identity", "percentage of sequence identity", and "substantial identity". A "reference sequence" is a defined sequence used as a basis for a sequence comparison; a reference sequence may be a subset of a larger sequence, for example, as a segment of a full-length cDNA or gene sequence given in a sequence listing, or may comprise a complete cDNA or gene sequence. Generally, a reference sequence is at least 20 nucleotides in length, frequently at least 25 nucleotides in length, and often at least 50 or 100 nucleotides in length. Since two polynucleotides may each (1) comprise a sequence (i.e., a portion of the complete polynucleotide sequence) that is similar between the two polynucleotides, and (2) may further comprise a sequence that is divergent between the two polynucleotides, sequence comparisons between two (or more) polynucleotides are typically performed by comparing sequences of the two polynucleotides over a "comparison window" to identify and compare local regions of sequence similarity.

A "comparison window", as used herein, refers to a conceptual segment of at least 20 contiguous nucleotides and wherein the portion of the

polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) of 20 percent or less as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences.

Methods of alignment of sequences for comparison are well known in the art. Thus, the determination of percent identity between any two sequences can be accomplished using a mathematical algorithm. Preferred, non-limiting examples of such mathematical algorithms are the algorithm of Myers and Miller (1988); the local homology algorithm of Smith and Waterman (1981); the homology alignment algorithm of Needleman and Wunsch (1970); the search-for-similarity-method of Pearson and Lipman (1988); the algorithm of Karlin and Altschul (1990), modified as in Karlin and Altschul (1993).

Computer implementations of these mathematical algorithms can be utilized for comparison of sequences to determine sequence identity. Such implementations include, but are not limited to: ClustalW (available, e.g., at http://www.ebi.ac.uk/clustalw/); the ALIGN program (Version 2.0) and GAP, BESTFIT, BLAST, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Version 8. Alignments using these programs can be performed using the default parameters. The CLUSTAL program is well described by Higgins et al. (1988); Higgins et al. (1989); Corpet et al. (1988); Huang et al. (1992); and Pearson et al. (1994). The ALIGN program is based on the algorithm of Myers and Miller, *supra*. The BLAST programs of Altschul et al. (1990), are based on the algorithm of Karlin and Altschul *supra*. To obtain gapped alignments for comparison purposes, Gapped BLAST (in BLAST 2.0) can be utilized as described in Altschul et al. (1997). Alternatively, PSI-BLAST (in BLAST 2.0) can be used to perform an iterated search that detects distant relationships between molecules. See Altschul et al., *supra*. When utilizing BLAST, Gapped BLAST, PSI-BLAST, the default parameters of the respective programs (e.g. BLASTN for nucleotide sequences, BLASTX for proteins) can be used. See http://www.ncbi.nlm.nih.gov. Alignment may also be performed manually by inspection

The term "sequence identity" means that two polynucleotide sequences are identical (i.e., on a nucleotide-by-nucleotide basis) over the window of

comparison. The term "percentage of sequence identity" means that two polynucleotide sequences are identical (i.e., on a nucleotide-by-nucleotide basis) for the stated proportion of nucleotides over the window of comparison. The term "percentage of sequence identity" is calculated by comparing two optimally aligned sequences over the window of comparison, determining the number of positions at which the identical nucleic acid base (e.g., A, T, C, G, U, or I) occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison (i.e., the window size), and multiplying the result by 100 to yield the percentage of sequence identity. The terms "substantial identity" as used herein denote a characteristic of a polynucleotide sequence, wherein the polynucleotide comprises a sequence that has at least 60%, preferably at least 65%, more preferably at least 70%, up to about 85%, and even more preferably at least 90 to 95%, more usually at least 99%, sequence identity as compared to a reference sequence over a comparison window of at least 20 nucleotide positions, frequently over a window of at least 20-50 nucleotides, and preferably at least 300 nucleotides, wherein the percentage of sequence identity is calculated by comparing the reference sequence to the polynucleotide sequence which may include deletions or additions which total 20 percent or less of the reference sequence over the window of comparison. The reference sequence may be a subset of a larger sequence.

As applied to polypeptides, the term "substantial identity" means that two peptide sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default gap weights, share at least about 85% sequence identity, preferably at least about 90% sequence identity, more preferably at least about 95 % sequence identity, and most preferably at least about 99 % sequence identity.

Synthetic Nucleotide Sequences and Methods of the Invention

The invention provides compositions comprising synthetic nucleotide sequences, as well as methods for preparing those sequences which yield synthetic nucleotide sequences that are efficiently expressed as a polypeptide or protein with desirable characteristics including reduced inappropriate or

unintended transcription characteristics, or do not result in inappropriate or unintended transcription characteristics, when present in a particular cell type.

Natural selection is the hypothesis that genotype-environment interactions occurring at the phenotypic level lead to differential reproductive success of individuals and hence to modification of the gene pool of a population. It is generally accepted that the amino acid sequence of a protein found in nature has undergone optimization by natural selection. However, amino acids exist within the sequence of a protein that do not contribute significantly to the activity of the protein and these amino acids can be changed to other amino acids with little or no consequence. Furthermore, a protein may be useful outside its natural environment or for purposes that differ from the conditions of its natural selection. In these circumstances, the amino acid sequence can be synthetically altered to better adapt the protein for its utility in various applications.

Likewise, the nucleic acid sequence that encodes a protein is also optimized by natural selection. The relationship between coding DNA and its transcribed RNA is such that any change to the DNA affects the resulting RNA. Thus, natural selection works on both molecules simultaneously. However, this relationship does not exist between nucleic acids and proteins. Because multiple codons encode the same amino acid, many different nucleotide sequences can encode an identical protein. A specific protein composed of 500 amino acids can theoretically be encoded by more than 10^{150} different nucleic acid sequences.

Natural selection acts on nucleic acids to achieve proper encoding of the corresponding protein. Presumably, other properties of nucleic acid molecules are also acted upon by natural selection. These properties include codon usage frequency, RNA secondary structure, the efficiency of intron splicing, and interactions with transcription factors or other nucleic acid binding proteins. These other properties may alter the efficiency of protein translation and the resulting phenotype. Because of the redundant nature of the genetic code, these other attributes can be optimized by natural selection without altering the corresponding amino acid sequence.

Under some conditions, it is useful to synthetically alter the natural nucleotide sequence encoding a protein to better adapt the protein for alternative

applications. A common example is to alter the codon usage frequency of a gene when it is expressed in a foreign host. Although redundancy in the genetic code allows amino acids to be encoded by multiple codons, different organisms favor some codons over others. The codon usage frequencies tend to differ most for organisms with widely separated evolutionary histories. It has been found that when transferring genes between evolutionarily distant organisms, the efficiency of protein translation can be substantially increased by adjusting the codon usage frequency (see U.S. Patent Nos. 5,096,825, 5,670,356 and 5,874,304).

In one embodiment, the sequence of a reporter gene is modified as the codon usage of reporter genes often does not correspond to the optimal codon usage of the experimental cells. In another embodiment, the sequence of a reporter gene is modified to remove regulatory sequences such as those which may alter expression of the reporter gene or a linked gene. Examples include β-galactosidase (β-*gal*) and chloramphenicol acetyltransferase (*cat*) reporter genes that are derived from *E. coli* and are commonly used in mammalian cells; the β-glucuronidase (*gus*) reporter gene that is derived from *E. coli* and commonly used in plant cells; the firefly luciferase (*luc*) reporter gene that is derived from an insect and commonly used in plant and mammalian cells; and the *Renilla* luciferase, and green fluorescent protein (*gfp*) reporter genes which are derived from coelenterates and are commonly used in plant and mammalian cells. To achieve sensitive quantitation of reporter gene expression, the activity of the gene product must not be endogenous to the experimental host cells. Thus, reporter genes are usually selected from organisms having unique and distinctive phenotypes. Consequently, these organisms often have widely separated evolutionary histories from the experimental host cells.

Previously, to create genes having a more optimal codon usage frequency but still encoding the same gene product, a synthetic nucleic acid sequence was made by replacing existing codons with codons that were generally more favorable to the experimental host cell (see U.S. Patent Nos. 5,096,825, 5,670,356 and 5,874,304.) The result was a net improvement in codon usage frequency of the synthetic gene. However, the optimization of other attributes was not considered and so these synthetic genes likely did not reflect genes optimized by natural selection.

In particular, improvements in codon usage frequency are intended only for optimization of a RNA sequence based on its role in translation into a protein. Thus, previously described methods did not address how the sequence of a synthetic gene affects the role of DNA in transcription into RNA. Most notably, consideration had not been given as to how transcription factors may interact with the synthetic DNA and consequently modulate or otherwise influence gene transcription. For genes found in nature, the DNA would be optimally transcribed by the native host cell and would yield an RNA that encodes a properly folded gene product. In contrast, synthetic genes have previously not been optimized for transcriptional characteristics. Rather, this property has been ignored or left to chance.

This concern is important for all genes, but particularly important for reporter genes, which are most commonly used to quantitate transcriptional behavior in the experimental host cells, and vector backbone sequences for genes. Hundreds of transcription factors have been identified in different cell types under different physiological conditions, and likely more exist but have not yet been identified. All of these transcription factors can influence the transcription of an introduced gene or sequences linked thereo. A useful synthetic reporter gene or vector backbone of the invention has a minimal risk of influencing or perturbing intrinsic transcriptional characteristics of the host cell because the structure of that gene or vector backbone has been altered. A particularly useful synthetic reporter gene or vector backbone will have desirable characteristics under a new set and/or a wide variety of experimental conditions. To best achieve these characteristics, the structure of the synthetic gene or synthetic vector backbone should have minimal potential for interacting with transcription factors within a broad range of host cells and physiological conditions. Minimizing potential interactions between a reporter gene or vector backbone and a host cell's endogenous transcription factors increases the value of a reporter gene or vector backbone by reducing the risk of inappropriate transcriptional characteristics of the gene or vector backbone within a particular experiment, increasing applicability of the gene or vector backbone in various environments, and increasing the acceptance of the resulting experimental data.

In contrast, a reporter gene comprising a native nucleotide sequence, based on a genomic or cDNA clone from the original host organism, or a vector backbone comprising native sequences found in one or a variety of different organisms, may interact with transcription factors when present in an exogenous host. This risk stems from two circumstances. First, the native nucleotide sequence contains sequences that were optimized through natural selection to influence gene transcription within the native host organism. However, these sequences might also influence transcription when the sequences are present in exogenous hosts, i.e., out of context, thus interfering with its performance as a reporter gene or vector backbone. Second, the nucleotide sequence may inadvertently interact with transcription factors that were not present in the native host organism, and thus did not participate in its natural selection. The probability of such inadvertent interactions increases with greater evolutionary separation between the experimental cells and the native organism of the reporter gene or vector backbone.

These potential interactions with transcription factors would likely be disrupted when using a synthetic reporter gene having alterations in codon usage frequency. However, a synthetic reporter gene sequence, designed by choosing codons based only on codon usage frequency, or randomly replacing sequences or randomly juxtaposing sequences in a vector backbone, is likely to contain other unintended transcription factor binding sites since the resulting sequence has not been subjected to the benefit of natural selection to correct inappropriate transcriptional activities. Inadvertent interactions with transcription factors could also occur whenever an encoded amino acid sequence is artificially altered, e.g., to introduce amino acid substitutions. Similarly, these changes have not been subjected to natural selection, and thus may exhibit undesired characteristics.

Thus, the invention provides a method for preparing synthetic nucleotide sequences that reduce the risk of undesirable interactions of the nucleotide sequence with transcription factors and other trans-acting factors when expressed in a particular host cell, thereby reducing inappropriate or unintended characteristics. Preferably, the method yields synthetic genes containing improved codon usage frequencies for a particular host cell and with a reduced

occurrence of regulatory sequences such as transcription factor binding sites and/or vector backbone sequences with a reduced occurrence of regulatory sequences. The invention also provides a method of preparing synthetic genes containing improved codon usage frequencies with a reduced occurrence of transcription factor binding sites and additional beneficial structural attributes. Such additional attributes include the absence of inappropriate RNA splicing junctions, poly(A) addition signals, undesirable restriction enzyme recognition sites, ribosomal binding sites, and/or secondary structural motifs such as hairpin loops.

In one embodiment, a parent nucleic acid sequence encoding a polypeptide is optimized for expression in a particular cell. For example, the nucleic acid sequence is optimized by replacing codons in the wild-type sequence with codons which are preferentially employed in a particular (selected) cell, which codon replacement also reduces the number of regulatory sequences. Preferred codons have a relatively high codon usage frequency in a selected cell, and preferably their introduction results in the introduction of relatively few regulatory sequences such as transcription factor binding sites, and relatively few other undesirable structural attributes. Thus, the optimized nucleotide sequence may have an improved level of expression due to improved codon usage frequency, and a reduced risk of inappropriate transcriptional behavior due to a reduced number of undesirable transcription regulatory sequences. In another embodiment, a parent vector backbone sequence is altered to remove regulatory sequences and optionally restriction endonuclease sites, and optionally retain or add other desirable characteristics, e.g., the presence of one or more stop codons in one or more reading frames, one or more poly(A) sites, and/or restriction endonuclease sites.

The invention may be employed with any nucleic acid sequence, e.g., a native sequence such as a cDNA or one that has been manipulated *in vitro*. Exemplary genes include, but are not limited to, those encoding lactamase (β-gal), neomycin resistance (Neo), hygromycin resistance (Hyg), puromycin resistance (Puro), ampicillin resistance (Amp), CAT, GUS, galactopyranoside, GFP, xylosidase, thymidine kinase, arabinosidase, luciferase and the like. As used herein, a "reporter gene" is a gene that imparts a distinct phenotype to cells

expressing the gene and thus permits cells having the gene to be distinguished from cells that do not have the gene. Such genes may encode either a selectable or screenable polypeptide, depending on whether the marker confers a trait which one can 'select' for by chemical means, i.e., through the use of a selective agent (e.g., a herbicide, antibiotic, or the like), or whether it is simply a "reporter" trait that one can identify through observation or testing, i.e., by 'screening'. Included within the terms selectable or screenable marker genes are also genes which encode a "secretable marker" whose secretion can be detected as a means of identifying or selecting for transformed cells. Examples include markers that encode a secretable antigen that can be identified by antibody interaction, or even secretable enzymes which can be detected by their catalytic activity. Secretable proteins fall into a number of classes, including small, diffusible proteins detectable, e.g., by ELISA, and proteins that are inserted or trapped in the cell membrane.

Elements of the present disclosure are exemplified in detail through the use of particular genes and vector backbone sequences. Of course, many examples of suitable genes and vector backbones are known to the art and can be employed in the practice of the invention. Therefore, it will be understood that the following discussion is exemplary rather than exhaustive. In light of the techniques disclosed herein and the general recombinant techniques that are known in the art, the present invention renders possible the alteration of any gene or vector backbone sequence.

Exemplary genes include, but are not limited to, a *neo* gene, a *puro* gene, an *amp* gene, a β-gal gene, a *gus* gene, a *cat* gene, a *gpt* gene, a *hyg* gene, a *hisD* gene, a *ble* gene, a *mprt* gene, a *bar* gene, a nitrilase gene, a mutant acetolactate synthase gene (ALS) or acetoacid synthase gene (AAS), a methotrexate-resistant *dhfr* gene, a dalapon dehalogenase gene, a mutated anthranilate synthase gene that confers resistance to 5-methyl tryptophan (WO 97/26366), an R-locus gene, a β-lactamase gene, a *xyl*E gene, an α-amylase gene, a tyrosinase gene, a luciferase (*luc*) gene (e.g., a *Renilla reniformis* luciferase gene, a firefly luciferase gene, or a click beetle luciferase (*Pyrophorus plagiophthalamus* gene), an aequorin gene, or a fluorescent protein gene.

The method of the invention can be performed by, although it is not limited to, a recursive process. The process includes assigning preferred codons to each amino acid in a target molecule, e.g., a native nucleotide sequence, based on codon usage in a particular species, identifying potential transcription regulatory sequences such as transcription factor binding sites in the nucleic acid sequence having preferred codons, e.g., using a database of such binding sites, optionally identifying other undesirable sequences, and substituting an alternative codon (i.e., encoding the same amino acid) at positions where undesirable transcription factor binding sites or other sequences occur. For codon distinct versions, alternative preferred codons are substituted in each version. If necessary, the identification and elimination of potential transcription factor or other undesirable sequences can be repeated until a nucleotide sequence is achieved containing a maximum number of preferred codons and a minimum number of undesired sequences including transcription regulatory sequences or other undesirable sequences. Also, optionally, desired sequences, e.g., restriction enzyme recognition sites, can be introduced. After a synthetic nucleotide sequence is designed and constructed, its properties relative to the parent nucleic acid sequence can be determined by methods well known to the art. For example, the expression of the synthetic and target nucleic acids in a series of vectors in a particular cell can be compared.

Thus, generally, the method of the invention comprises identifying a target nucleic acid sequence, and a host cell of interest, for example, a plant (dicot or monocot), fungus, yeast or mammalian cell. Preferred host cells are mammalian host cells such as CHO, COS, 293, Hela, CV-1 and NIH3T3 cells. Based on preferred codon usage in the host cell(s) and, optionally, low codon usage in the host cell(s), e.g., high usage mammalian codons and low usage *E. coli* and mammalian codons, codons to be replaced are determined. Concurrent, subsequent or prior to selecting codons to be replaced, desired and undesired sequences, such as undesired transcriptional regulatory sequences, in the target sequence are identified. These sequences, including transcriptional regulatory sequences and restriction endonuclease sites, can be identified using databases and software such as TRANSFAC® (Transcription Factor Database, http://www.gene-regulation.com/), Match™ (http://www.gene-regulation.com/),

MatInspector (Genomatix, http://www.genomatix.de), EPD (Eukaryotic Promoter Database, http://www.epd.isb-sib.ch/), REBASE® (Restriction Enzyme Database, NEB, http://rebase.neb.com), TESS (Transcription Element Search System, http://www.cbil.upenn.edu/tess/), MAR-Wiz (Futuresoft, http://www.futuresoft.org), Lasergene® (DNASTAR, http://www.dnastar.com), Vector NTI™ (Invitrogen, http://www.invitrogen.com), and Sequence Manipulation Suite (http://www.bioinformatics.org/SMS/index.html). Links to other databases and sequence analysis software are listed at http://www.expasy.org/alinks.html. After one or more sequences are identified, the modification(s) may be introduced. Once a desired synthetic nucleotide sequence is obtained, it can be prepared by methods well known to the art (such as nucleic acid amplification reactions with overlapping primers), and its structural and functional properties compared to the target nucleic acid sequence, including, but not limited to, percent homology, presence or absence of certain sequences, for example, restriction sites, percent of codons changed (such as an increased or decreased usage of certain codons) and/or expression rates.

As described below, the method was used to create synthetic reporter genes encoding firefly luciferases and selectable polypeptides, and synthetic sequences for vector backbones. Synthetic sequences may support greater levels of expression and/or reduced aberrant expression than the corresponding native or parent sequenes for the protein. The native and parent sequences may demonstrate anomalous transcription characteristics when expressed in mammalian cells, which are likely not evident in the synthetic sequences.

Exemplary Uses of the Synthetic Nucleotide Sequences

The synthetic genes of the invention preferably encode the same proteins as their native counterpart (or nearly so), but have improved codon usage while being largely devoid of regulatory elements in the coding (it is recognized that a small number of amino acid changes may be desired to enhance a property of the native counterpart protein, e.g. to enhance luminescence of a luciferase) and noncoding regions. This increases the level of expression of the protein the synthetic gene encodes and reduces the risk of anomalous expression of the protein. For example, studies of many important events of gene regulation,

which may be mediated by weak promoters, are limited by insufficient reporter signals from inadequate expression of the reporter proteins. Also, the use of some selectable markers may be limited by the expression of that marker in an exogenous cell. Thus, synthetic selectable marker genes which have improved codon usage for that cell, and have a decrease in other undesirable sequences, (e.g., transcription factor binding sites), can permit the use of those markers in cells that otherwise were undesirable as hosts for those markers.

Promoter crosstalk is another concern when a co-reporter gene is used to normalize transfection efficiencies. With the enhanced expression of synthetic genes, the amount of DNA containing strong promoters can be reduced, or DNA containing weaker promoters can be employed, to drive the expression of the co-reporter. In addition, there may be a reduction in the background expression from the synthetic reporter genes of the invention. This characteristic makes synthetic reporter genes more desirable by minimizing the sporadic expression from the genes and reducing the interference resulting from other regulatory pathways.

The use of reporter genes in imaging systems, which can be used for *in vivo* biological studies or drug screening, is another use for the synthetic genes of the invention. Due to their increased level of expression, the protein encoded by a synthetic gene is more readily detectable by an imaging system. In fact, using a synthetic *Renilla* luciferase gene, luminescence in transfected CHO cells was detected visually without the aid of instrumentation.

In addition, the synthetic genes may be used to express fusion proteins, for example fusions with secretion leader sequences or cellular localization sequences, to study transcription in difficult-to-transfect cells such as primary cells, and/or to improve the analysis of regulatory pathways and genetic elements. Other uses include, but are not limited to, the detection of rare events that require extreme sensitivity (e.g., studying RNA recoding), use with IRES, to improve the efficiency of *in vitro* translation or *in vitro* transcription-translation coupled systems such as TnT (Promega Corp., Madison, WI), study of reporters optimized to different host organisms (e.g., plants, fungus, and the like), use of multiple genes as co-reporters to monitor drug toxicity, as reporter molecules in multiwell assays, and as reporter molecules in drug screening with the advantage

of minimizing possible interference of reporter signal by different signal transduction pathways and other regulatory mechanisms.

Additionally, uses for the synthetic nucleotide sequences of the invention include fluorescence activated cell sorting (FACS), fluorescent microscopy, to detect and/or measure the level of gene expression *in vitro* and *in vivo*, (e.g., to determine promoter strength), subcellular localization or targeting (fusion protein), as a marker, in calibration, in a kit (e.g., for dual assays), for *in vivo* imaging, to analyze regulatory pathways and genetic elements, and in multi-well formats.

Further, although reporter genes are widely used to measure transcription events, their utility can be limited by the fidelity and efficiency of reporter expression. For example, in U.S. Patent No. 5,670,356, a firefly luciferase gene (referred to as luc+) was modified to improve the level of luciferase expression. While a higher level of expression was observed, it was not determined that higher expression had improved regulatory control.

The invention will be further described by the following nonlimiting examples. In particular, the synthetic nucleic acid molecules of the invention may be derived by other methods as well as by variations on the methods described herein.

Example 1

Synthetic Click Beetle (RD and GR) Luciferase Nucleic Acid Molecules

Luc*Ppl*YG is a wild-type click beetle luciferase that emits yellow-green luminescence (Wood, 1989). A mutant of Luc*Ppl*YG named YG#81-6G01 was envisioned. YG#81-6G01 lacks a peroxisome targeting signal, has a lower K_M for luciferin and ATP, has increased signal stability and increased temperature stability when compared to the wild type (PCT/WO9914336). YG #81-6G01 was mutated to emit green luminescence by changing Ala at position 224 to Val (A224V is a green-shifting mutation), or to emit red luminescence by simultaneously introducing the amino acid substitutions A224H, S247H, N346I, and H348Q (red-shifting mutation set) (PCT/WO9518853)

Using YG #81-6G01 as a parent gene, two synthetic gene sequences were designed. One codes for a luciferase emitting green luminescence (GR) and one

for a luciferase emitting red luminescence (RD). Both genes were designed to 1) have optimized codon usage for expression in mammalian cells, 2) have a reduced number of transcriptional regulatory sites including mammalian transcription factor binding sites, splice sites, poly(A) sites and promoters, as well as prokaryotic (*E. coli*) regulatory sites, 3) be devoid of unwanted restriction sites, e.g., those which are likely to interfere with standard cloning procedures, and 4) have a low DNA sequence identity compared to each other in order to minimize genetic rearrangements when both are present inside the same cell. In addition, desired sequences, e.g., a Kozak sequence or restriction enzyme recognition sites, may be identified and introduced.

Not all design criteria could be met equally well at the same time. The following priority was established for reduction of transcriptional regulatory sites: elimination of transcription factor (TF) binding sites received the highest priority, followed by elimination of splice sites and poly(A) sites, and finally prokaryotic regulatory sites. When removing regulatory sites, the strategy was to work from the lesser important to the most important to ensure that the most important changes were made last. Then the sequence was rechecked for the appearance of new lower priority sites and additional changes made as needed. Thus, the process for designing the synthetic GR and RD gene sequences, using computer programs described herein, involved 5 optionally iterative steps that are detailed below

1. Optimized codon usage and changed A224V to create GRver1, separately changed A224H, S247H, H348Q and N346I to create RDver1. These particular amino acid changes were maintained throughout all subsequent manipulations to the sequence.
2. Removed undesired restriction sites, prokaryotic regulatory sites, splice sites, poly(A) sites thereby creating GRver2 and RDver2.
3. Removed transcription factor binding sites (first pass) and removed any newly created undesired sites as listed in step 2 above thereby creatingGRver3 and RDver3.
4. Removed transcription factor binding sites created by step 3 above (second pass) and removed any newly created undesired sites as listed in step 2 above thereby creating GRver4 and RDver4.

5. Removed transcription factor binding sites created by step 4 above (third Pass) and confirmed absence of sites listed in step 2 above thereby creating GRver5 and RDver5.
6. Constructed the actual genes by PCR using synthetic oligonucleotides corresponding to fragments of GRver5 and RDver5 designed sequences thereby creating GR6 and RD7. GR6, upon sequencing was found to have the serine residue at amino acid position 49 mutated to an asparagine and the proline at amino acid position 230 mutated to a serine (S49N, P230S). RD7, upon sequencing was found to have the histidine at amino acid position 36 mutated to a tyrosine (H36Y). These changes occurred during the PCR process.
4. The mutations described in step 6 above (S49N, P230S for GR6 and H36Y for RD7) were reversed to create GRver5.1 and RDver5.1.
5. RDver5.1 was further modified by changing the arginine codon at position 351 to a glycine codon (R351G) thereby creating RDver5.2 with improved spectral properties compared to RDver5.1.
6. RDver5.2 was further mutated to increase luminescence intensity thereby creating RD156-1H9 which encodes four additional amino acid changes (M2I, S349T, K488T, E538V) and three silent single base changes (see U.S. application Serial No. 09/645,706, filed August 24, 2000, the disclosure of which is incorporated by reference herein).

1. Optimize codon usage and introduce mutations determining luminescence color

The starting gene sequence for this design step was YG #81-6G01.

a) Optimize codon usage:

The strategy was to adapt the codon usage for optimal expression in human cells and at the same time to avoid *E. coli* low-usage codons. Based on these requirements, the best two codons for expression in human cells for all amino acids with more than two codons were selected (see Wada et al., 1990). In the selection of codon pairs for amino acids with six codons, the selection was biased towards pairs that have the largest number of mismatched bases to allow

design of GR and RD genes with minimum sequence identity (codon distinction):

Arg: CGC/CGT	Leu: CTG/TTG	Ser: TCT/AGC
Thr: ACC/ACT	Pro: CCA/CCT	Ala: GCC/GCT
Gly: GGC/GGT	Val: GTC/GTG	Ile: ATC/ATT

Based on this selection of codons, two gene sequences encoding the YG#81-6G01 luciferase protein sequence were computer generated. The two genes were designed to have minimum DNA sequence identity and at the same time closely similar codon usage. To achieve this, each codon in the two genes was replaced by a codon from the limited list described above in an alternating fashion (e.g., $Arg_{(n)}$ is CGC in gene 1 and CGT in gene 2, $Arg_{(n+1)}$ is CGT in gene 1 and CGC in gene 2).

For subsequent steps in the design process it was anticipated that changes had to be made to this limited optimal codon selection in order to meet other design criteria, however, the following low-usage codons in mammalian cells were not used unless needed to meet criteria of higher priority:

Arg: CGA	Leu: CTA	Ser: TCG
Pro: CCG	Val: GTA	Ile: ATA

Also, the following low-usage codons in *E. coli* were avoided when reasonable (note that 3 of these match the low-usage list for mammalian cells):

Arg: CGA/CGG/AGA/AGG

Leu: CTA	Pro: CCC	Ile: ATA

b) Introduce mutations determining luminescence color:

Into one of the two codon-optimized gene sequences was introduced the single green-shifting mutation and into the other were introduced the 4 red-shifting mutations as described above.

The two output sequences from this first design step were named GRver1 (version 1 GR) and RDver1 (version 1 RD). Their DNA sequences are 63% identical (594 mismatches), while the proteins they encode differ only by the 4 amino acids that determine luminescence color (see Figures 2 and 3 for an alignment of the DNA and protein sequences).

Tables 1 and 2 show, as an example, the codon usage for valine and leucine in human genes, the parent gene YG#81-6G01, the codon-optimized

synthetic genes GRver1 and RDver1, as well as the final versions of the synthetic genes after completion of step 5 in the design process (GRver5 and RDver5).

Table 1: Valine

Codon	Human	Parent	GR ver1	RD ver1	GR ver5	RD ver5
GTA	4	13	0	0	1	1
GTC	**13**	**4**	**25**	**24**	**21**	**26**
GTG	**24**	**12**	**25**	**25**	**25**	**17**
GTT	9	20	0	0	3	5

Table 2: Leucine

Codon	Human	Parent	GR ver1	RD ver1	GR ver5	RD ver5
CTA	3	5	0	0	0	0
CTC	12	4	0	1	12	11
CTG	**24**	**4**	**28**	**27**	**19**	**18**
CTT	6	12	0	0	1	1
TTA	3	17	0	0	0	0
TTG	**6**	**13**	**27**	**27**	**23**	**25**

2. Remove undesired restriction sites, prokaryotic regulatory sites, splice sites and poly(A) sites

The starting gene sequences for this design step were GRver1 and RDver1.

a) Remove undesired restriction sites:

To check for the presence and location of undesired restriction sites, the sequences of both synthetic genes were compared against a database of restriction enzyme recognition sequences (REBASE ver.712, http://www.neb.com/rebase) using standard sequence analysis software (GenePro ver 6.10, Riverside Scientific Ent.).

Specifically, the following restriction enzymes were classified as undesired:

- *Bam*H I, *Xho* I, *Sfi* I, *Kpn* I, *Sac* I, *Mlu* I, *Nhe* I, *Sma* I, *Xho* I, *Bgl* II, *Hind* III, *Nco* I, *Nar* I, *Xba* I, *Hpa* I, *Sal* I,
- other cloning sites commonly used: *Eco*R I, *Eco*R V, *Cla* I,
- eight-base cutters (commonly used for complex constructs),
- *Bst*E II (to allow N-terminal fusions),
- *Xcm* I (can generate A/T overhang used for T-vector cloning).

To eliminate undesired restriction sites when found in a synthetic gene, one or more codons of the synthetic gene sequence were altered in accordance with the codon optimization guidelines described in 1a above.

b) Remove prokaryotic (*E. coli*) regulatory sequences:

To check for the presence and location of prokaryotic regulatory sequences, the sequences of both synthetic genes were searched for the presence of the following consensus sequences using standard sequence analysis software (GenePro):

- TATAAT (-10 Pribnow box of promoter)
- AGGA or GGAG (ribosome binding site; only considered if paired with a methionine codon 12 or fewer bases downstream).

To eliminate such regulatory sequences when found in a synthetic gene, one or more codons of the synthetic gene at sequence were altered in accordance with the codon optimization guidelines described in 1a above.

c) Remove splice sites:

To check for the presence and location of splice sites, the DNA strand corresponding to the primary RNA transcript of each synthetic gene was searched for the presence of the following consensus sequences (see Watson et al., 1983) using standard sequence analysis software (GenePro):

- splice donor site: AG | GTRAGT (exon | intron), the search was performed for AGGTRAG and the lower stringency GGTRAGT;
- splice acceptor site: $(Y)_nNCAG | G$ (intron | exon), the search was performed with $n = 1$.

To eliminate splice sites found in a synthetic gene, one or more codons of the synthetic gene sequence were altered in accordance with the codon optimization guidelines described in 1a above. Splice acceptor sites were generally difficult to eliminate in one gene without introducing them into the other gene because they tended to contain one of the two only Gln codons (CAG); they were removed by placing the Gln codon CAA in both genes at the expense of a slightly increased sequence identity between the two genes.

d) Remove poly(A) sites:

To check for the presence and location of poly(A) sites, the sequences of both synthetic genes were searched for the presence of the following consensus sequence using standard sequence analysis software (GenePro):

- AATAAA.

To eliminate each poly(A) addition site found in a synthetic gene, one or more codons of the synthetic gene sequence were altered in accordance with the codon optimization guidelines described in 1a above. The two output sequences from this second design step were named GRver2 and RDver2. Their DNA sequences are 63% identical (590 mismatches).

3. Remove transcription factor (TF) binding sites, then repeat steps 2 a-d

The starting gene sequences for this design step were GRver2 and RDver2.

To check for the presence, location and identity of potential TF binding sites, the sequences of both synthetic genes were used as query sequences to search a database of transcription factor binding sites (TRANSFAC v3.2). The TRANSFAC database (http://transfac.gbf.de/TRANSFAC/index:html) holds information on gene regulatory DNA sequences (TF binding sites) and proteins (TFs) that bind to and act through them. The SITE table of TRANSFAC Release 3.2 contains 4,401 entries of individual (putative) TF binding sites (including TF binding sites in eukaryotic genes, in artificial sequences resulting from mutagenesis studies and *in vitro* selection procedures based on random oligonucleotide mixtures or specific theoretical considerations, and consensus binding sequences (from Faisst and Meyer, 1992).

The software tool used to locate and display these TF binding sites in the synthetic gene sequences was TESS (Transcription Element Search Software, http://agave.humgen.upenn.edu/tess/index.html). The filtered string-based search option was used with the following user-defined search parameters:

- Factor Selection Attribute: Organism Classification
- Search Pattern: Mammalia
- Max. Allowable Mismatch %: 0
- Min. element length: 5

- Min. log-likelihood: 10

This parameter selection specifies that only mammalian TF binding sites (approximately 1,400 of the 4,401 entries in the database) that are at least 5 bases long will be included in the search. It further specifies that only TF binding sites that have a perfect match in the query sequence and a minimum log likelihood (LLH) score of 10 will be reported. The LLH scoring method assigns 2 to an unambiguous match, 1 to a partially ambiguous match (e.g., A or T match W) and 0 to a match against 'N'. For example, a search with parameters specified above would result in a "hit" (positive result or match) for TATAA (SEQ ID NO:50) (LLH = 10), STRATG (SEQ ID NO:51) (LLH = 10), and MTTNCNNMA (SEQ ID NO:52) (LLH = 10) but not for TRATG (SEQ ID NO: 53) (LLH = 9) if these four TF binding sites were present in the query sequence. A lower stringency test was performed at the end of the design process to re-evaluate the search parameters.

When TESS was tested with a mock query sequence containing known TF binding sites it was found that the program was unable to report matches to sites ending with the 3' end of the query sequence. Thus, an extra nucleotide was added to the 3' end of all query sequences to eliminate this problem.

The first search for TF binding sites using the parameters described above found about 100 transcription factor binding sites (hits) for each of the two synthetic genes (GRver2 and RDver2). All sites were eliminated by changing one or more codons of the synthetic gene sequences in accordance with the codon optimization guidelines described in 1a above. However, it was expected that some these changes created new TF binding sites, other regulatory sites, and new restriction sites. Thus, steps 2 a-d were repeated as described, and 4 new restriction sites and 2 new splice sites were removed. The two output sequences from this third design step were named GRver3 and RDver3. Their DNA sequences are 66% identical (541 mismatches).

4. Remove new transcription factor (TF) binding sites, then repeat steps 2 a-d

The starting gene sequences for this design step were GRver3 and RDver3.

This fourth step is an iteration of the process described in step 3. The search for newly introduced TF binding sites yielded about 50 hits for each of the two synthetic genes. All sites were eliminated by changing one or more codons of the synthetic gene sequences in general accordance with the codon optimization guidelines described in 1a above. However, more high to medium usage codons were used to allow elimination of all TF binding sites. The lowest priority was placed on maintaining low sequence identity between the GR and RD genes. Then steps 2 a-d were repeated as described. The two output sequences from this fourth design step were named GRver4 and RDver4. Their DNA sequences are 68% identical (506 mismatches).

5. Remove new transcription factor (TF) binding sites, then repeat steps 2 a-d

The starting gene sequences for this design step were GRver4 and RDver4.

This fifth step is another iteration of the process described in step 3 above. The search for new TF binding sites introduced in step 4 yielded about 20 hits for each of the two synthetic genes. All sites were eliminated by changing one or more codons of the synthetic gene sequences in general accordance with the codon optimization guidelines described in 1a above. However, more high to medium usage codons were used (these are all considered "preferred") to allow elimination of all TF binding sites. The lowest priority was placed on maintaining low sequence identity between the GR and RD genes. Then steps 2 a-d were repeated as described. Only one acceptor splice site could not be eliminated. As a final step the absence of all TF binding sites in both genes as specified in step 3 was confirmed. The two output sequences from this fifth and last design step were named GRver5 and RDver5. Their DNA sequences are 69% identical (504 mismatches).

Additional evaluation of GRver5 and RDver5

a) Use lower stringency parameters for TESS:

The search for TF binding sites was repeated as described in step 3 above, but with even less stringent user-defined parameters:

- setting LLH to 9 instead of 10 did not result in new hits;

- setting LLH to 0 through 8 (incl.) resulted in hits for two additional sites, MAMAG (22 hits) and CTKTK (24 hits);
- setting LLH to 8 and the minimum element length to 4, the search yielded (in addition to the two sites above) different 4-base sites for AP-1, NF-1, and c-Myb that are shortened versions of their longer respective consensus sites which were eliminated in steps 3-5 above.

It was not realistic to attempt complete elimination of these sites without introduction of new sites, so no further changes were made.

b) Search different database:

The Eukaryotic Promoter Database (release 45) contains information about reliably mapped transcription start sites (1253 sequences) of eukaryotic genes. This database was searched using BLASTN 1.4.11 with default parameters (optimized to find nearly identical sequences rapidly; see Altschul et al, 1990) at the National Center for Biotechnology Information site (http://www.ncbi.nlm.nih.gov/cgi-bin/BLAST). To test this approach, a portion of pGL3-Control vector sequence containing the SV40 promoter and enhancer was used as a query sequence, yielding the expected hits to SV40 sequences. No hits were found when using the two synthetic genes as query sequences.

Summary of GRver5 and RDver5 synthetic gene properties

Both genes, which at this stage were still only "virtual" sequences in the computer, have a codon usage that strongly favors mammalian high-usage codons and minimizes mammalian and *E. coli* low-usage codons.

Both genes are also completely devoid of eukaryotic TF binding sites consisting of more than four unambiguous bases, donor and acceptor splice sites (one exception: GRver5 contains one splice acceptor site), poly(A) sites, specific prokaryotic (*E. coli*) regulatory sequences, and undesired restriction sites.

The gene sequence identity between GRver5 and RDver5 is only 69% (504 base mismatches) while their encoded proteins are 99% identical (4 amino acid mismatches). Their identity with the parent sequence YG#81-6G1 is 74% (GRver5) and 73% (RDver5). Their base composition is 49.9% GC (GRver5) and 49.5% GC (RDver5), compared to 40.2% GC for the parent YG#81-6G01.

Construction of synthetic genes

The two synthetic genes were constructed by assembly from synthetic oligonucleotides in a thermocycler followed by PCR amplification of the full-length genes (similar to Stemmer et al. (1995) Gene. 164, pp. 49-53). Unintended mutations that interfered with the design goals of the synthetic genes were corrected.

a) Design of synthetic oligonucleotides:

The synthetic oligonucleotides were mostly 40mers that collectively code for both complete strands of each designed gene (1,626 bp) plus flanking regions needed for cloning (1,950 bp total for each gene). The 5' and 3' boundaries of all oligonucleotides specifying one strand were generally placed in a manner to give an average offset/overlap of 20 bases relative to the boundaries of the oligonucleotides specifying the opposite strand.

The ends of the flanking regions of both genes matched the ends of the amplification primers (pRAMtailup: 5'-gtactgagacgacgccagcccaagcttaggcctgagtg SEQ ID NO:54, and pRAMtaildn: 5'-ggcatgagcgtgaactgactgaactagcggccgccgag SEQ ID NO:55) to allow cloning of the genes into our *E. coli* expression vector pRAM (WO99/14336).

A total of 183 oligonucleotides were designed: fifteen oligonucleotides that collectively encode the upstream and downstream flanking sequences and 168 oligonucleotides (4 x 42) that encode both strands of the two genes.

All 183 oligonucleotides were run through the hairpin analysis of the OLIGO software (OLIGO 4.0 Primer Analysis Software © 1989-1991 by Wojciech Rychlik) to identify potentially detrimental intra-molecular loop formation. The guidelines for evaluating the analysis results were set according to recommendations of Dr. Sims (Sigma-Genosys Custom Gene Synthesis Department): oligos forming hairpins with $\Delta G < -10$ have to be avoided, those forming hairpins with $\Delta G \leq -7$ involving the 3' end of the oligonucleotide should also be avoided, while those with an overall $\Delta G \leq -5$ should not pose a problem for this application. The analysis identified 23 oligonucleotides able to form hairpins with a ΔG between -7.1 and -4.9. Of these, 5 had blocked or nearly blocked 3' ends (0-3 free bases) and were re-designed by removing 1-4 bases at their 3' end and adding it to the adjacent oligonucleotide.

The 40mer oligonucleotide covering the sequence complementary to the poly(A) tail had a very low complexity 3′ end (13 consecutive T bases). An additional 40mer was designed with a high complexity 3′ end but a consequently reduced overlap with one of its complementary oligonucleotides (11 instead of 20 bases) on the opposite strand.

Even though the oligonucleotides were designed for use in a thermocycler-based assembly reaction, they could also be used in a ligation-based protocol for gene construction. In this approach, the oligonucleotides are annealed in a pairwise fashion and the resulting short double-stranded fragments are ligated using the sticky overhangs. However, this would require that all oligonucleotides be phosphorylated.

b) Gene assembly and amplification

In a first step, each of the two synthetic genes was assembled in a separate reaction from 98 oligonucleotides. The total volume for each reaction was 50 µl:

0.5 µM oligonucleotides (= 0.25 pmoles of each oligo)
1.0 U *Taq* DNA polymerase
0.02 U *Pfu* DNA polymerase
2 mM $MgCl_2$
0.2 mM dNTPs (each)
0.1% gelatin
Cycling conditions: (94°C for 30 seconds, 52°C for 30 seconds, and 72°C for 30 seconds) x 55 cycles.

In a second step, each assembled synthetic gene was amplified in a separate reaction. The total volume for each reaction was 50 µl:

2.5 l assembly reaction
5.0 U *Taq* DNA polymerase
0.1 U *Pfu* DNA polymerase
1 M each primer (pRAMtailup, pRAMtaildn)
2 mM $MgCl_2$
0.2 mM dNTPs (each)
Cycling conditions: (94°C for 20 seconds, 65°C for 60 seconds, 72°C for 3 minutes) x 30 cycles.

The assembled and amplified genes were subcloned into the pRAM vector and expressed in *E. coli*, yielding 1-2% luminescent GR or RD clones. Five GR and five RD clones were isolated and analyzed further. Of the five GR clones, three had the correct insert size, of which one was weakly luminescent and one had an altered restriction pattern. Of the five RD clones, two had the correct size insert with an altered restriction pattern and one of those was weakly luminescent. Overall, the analysis indicated the presence of a large number of mutations in the genes, most likely the result of errors introduced in the assembly and amplification reactions.

c) Corrective assembly and amplification

To remove the large number of mutations present in the full-length synthetic genes we performed an additional assembly and amplification reaction for each gene using the proof-reading DNA polymerase *Tli*. The assembly reaction contained, in addition to the 98 GR or RD oligonucleotides, a small amount of DNA from the corresponding full-length clones with mutations described above. This allows the oligos to correct mutations present in the templates.

The following assembly reaction was performed for each of the synthetic genes. The total volume for each reaction was 50 µl:

0.5 µM oligonucleotides (= 0.25 pmoles of each oligo)
0.016 pmol plasmid (mix of clones with correct insert size)
2.5 U *Tli* DNA polymerase
2 mM $MgCl_2$
0.2 mM dNTPs (each)
0.1 % gelatin
Cycling conditions: 94°C for 30 seconds, then (94°C for 30 seconds, 52°C for 30 seconds, 72°C for 30 seconds) for 55 cycles, then 72°C for 5 minutes.

The following amplification reaction was performed on each of the assembly reactions. The total volume for each amplification reaction was 50 µl:

1-5 µl of assembly reaction
40 pmol each primer (pRAMtailup, pRAMtaildn)

2.5 U *Tli* DNA polymerase

2 mM $MgCl_2$

0.2 mM dNTPs (each)

Cycling conditions: 94°C for 30 seconds, then (94°C for 20 seconds, 65°C for 60 seconds and 72°C for 3 minutes) for 30 cycles, then 72°C for 5 minutes.

The genes obtained from the corrective assembly and amplification step were subcloned into the pRAM vector and expressed in *E. coli*, yielding 75% luminescent GR or RD clones. Forty-four GR and 44 RD clones were analyzed with the screening robot described in WO99/14336. The six best GR and RD clones were manually analyzed and one best GR and RD clone was selected (GR6 and RD7). Sequence analysis of GR6 revealed two point mutations in the coding region, both of which resulted in an amino acid substitution (S49N and P230S). Sequence analysis of RD7 revealed three point mutations in the coding region, one of which resulted in an amino acid substitution (H36Y). It was confirmed that none of the silent point mutations introduced any regulatory or restriction sites conflicting with the overall design criteria for the synthetic genes.

d) Reversal of unintended amino acid substitutions

The unintended amino acid substitutions present in the GR6 and RD7 synthetic genes were reversed by site-directed mutagenesis to match the GRver5 and RDver5 designed sequences, thereby creating GRver5.1 and RDver5.1. The DNA sequences of the mutated regions were confirmed by sequence analysis.

e) Improve spectral properties

The RDver5.1 gene was further modified to improve its spectral properties by introducing an amino change (R351G), thereby creating RDver5.2

pGL3 vectors with RD and GR genes

The parent click beetle luciferase YG#81-6G1 ("YG"), and the synthetic click beetle luciferase genes GRver5.1 ("GR"), RDver5.2 ("RD"), and RD156-1H9 were cloned into the four pGL3 reporter vectors (Promega Corp.):

- pGL3-Basic = no promoter, no enhancer
- pGL3-Control = SV40 promoter, SV40 enhancer

- pGL3-Enhancer = SV40 enhancer (3' to luciferase coding sequences)
- pGL3-Promoter = SV40 promoter.

The primers employed in the assembly of GR and RD synthetic genes facilitated the cloning of those genes into pRAM vectors. To introduce the genes into pGL3 vectors (Promega Corp., Madison, WI) for analysis in mammalian cells, each gene in a pRAM vector (pRAM RDver5.1, pRAM GRver5.1, and pRAM RD156-1H9) was amplified to introduce an *Nco* I site at the 5' end and an *Xba* I site at the 3' end of the gene. The primers for pRAM RDver5.1 and pRAM GRver5.1 were:

GR→5' GGA TCC CAT GGT GAA GCG TGA GAA 3' (SEQ ID NO:56) or

RD→5' GGA TCC CAT GGT GAA ACG CGA 3' (SEQ ID NO:57) and

5' CTA GCT TTT TTT TCT AGA TAA TCA TGA AGA C 3' (SEQ ID NO:58)

The primers for pRAM RD156-1H9 were:

5' GCG TAG CCA TGG TAA AGC GTG AGA AAA ATG TC 3' (SEQ ID NO: 59) and

5' CCG ACT CTA GAT TAC TAA CCG CCG GCC TTC ACC 3' (SEQ ID NO: 60)

The PCR included:

100 ng DNA plasmid
1 μM primer upstream
1 μM primer downstream
0.2 mM dNTPs
1X buffer (Promega Corp.)
5 units *Pfu* DNA polymerase (Promega Corp.)
Sterile nanopure H_2O to 50 μl

The cycling parameters were: 94°C for 5 minutes; (94°C for 30 seconds; 55°C for 1 minute; and 72°C for 3 minutes) x 15 cycles. The purified PCR product was digested with *Nco* I and *Xba* I, ligated with pGL3-control that was also digested with *Nco* I and *Xba* I, and the ligated products introduced to *E. coli*. To insert the luciferase genes into the other pGL3 reporter vectors (basic, promoter and enhancer), the pGL3-control vectors containing each of the luciferase genes was digested with *Nco* I and *Xba* I, ligated with other pGL3

vectors that also were digested with *Nco* I and *Xba* I, and the ligated products introduced to *E. coli*. Note that the polypeptide encoded by GRver5.1 and RDver5.1 (and RD156-1H9, see below) nucleic acid sequences in pGL3 vectors has an amino acid substitution at position 2 to valine as a result of the *Nco* I site at the initiation codon in the oligonucleotide.

Because of internal *Nco* I and *Xba* I sites, the native gene in YG #81-6G01 was amplified from a *Hind* III site upstream to a *Hpa* I site downstream of the coding region and which included flanking sequences found in the GR and RD clones. The upstream primer (5′-CAA AAA GCT TGG CAT TCC GGT ACT GTT GGT AAA GCC ACC ATG GTG AAG CGA GAG- 3′; SEQ ID NO:61) and a downstream primer (5′- CAA TTG TTG TTG TTA ACT TGT TTA TT -3′; SEQ ID NO:62) were mixed with YG#81-6G01 and amplified using the PCR conditions above. The purified PCR product was digested with *Nco* I and *Xba* I, ligated with pGL3-control that was also digested with *Hind* III and *Hpa* I, and the ligated products introduced into *E. coli*. To insert YG#81-6G01 into the other pGL3 reporter vectors (basic, promoter and enhancer), the pGL3-control vectors containing YG#81-6G01 were digested with *Nco* I and *Xba* I, ligated with the other pGL3 vectors that also were digested with *Nco* I and *Xba* I, and the ligated products introduced to *E. coli*. Note that the clone of YG#81-6G01 in the pGL3 vectors has a C instead of an A at base 786, which yields a change in the amino acid sequence at residue 262 from Phe to Leu. To determine whether the altered amino acid at position 262 affected the enzyme biochemistry, the clone of YG#81-6G01 was mutated to resemble the original sequence. Both clones were then tested for expression in *E. coli*, physical stability, substrate binding, and luminescence output kinetics. No significant differences were found.

Partially purified enzymes expressed from the synthetic genes and the parent gene were employed to determine Km for luciferin and ATP (see Table 3).

Table 3

Enzyme	K_M (LH_2)	K_M (ATP)
YG parent	2 μM	17 μM
GR	1.3 μM	25 μM
RD	24.5 μM	46 μM

In vitro eukaryotic transcription/translation reactions were also conducted using Promega's TNT T7 Quick system according to manufacturer's instructions. Luminescence levels were 1 to 37-fold and 1 to 77-fold higher (depending on the reaction time) for the synthetic GR and RD genes, respectively, compared to the parent gene (corrected for luminometer spectral sensitivity).

To test whether the synthetic click beetle luciferase genes and the wild type click beetle gene have improved expression in mammalian cells, each of the synthetic genes and the parent gene was cloned into a series of pGL3 vectors and introduced into CHO cells (Table 8). In all cases, the synthetic click beetle genes exhibited a higher expression than the native gene. Specifically, expression of the synthetic GR and RD genes was 1900-fold and 40-fold higher, respectively, than that of the parent (transfection efficiency normalized by comparison to native *Renilla* luciferase gene). Moreover, the data (basic versus control vector) show that the synthetic genes have reduced basal level transcription.

Further, in experiments with the enhancer vector where the percentage of activity in reference to the control is compared between the native and synthetic gene, the data showed that the synthetic genes have reduced risk of anomalous transcription characteristics. In particular, the parent gene appeared to contain one or more internal transcriptional regulatory sequences that are activated by the enhancer in the vector, and thus is not suitable as a reporter gene while the synthetic GR and RD genes showed a clean reporter response (transfection efficiency normalized by comparison to native *Renilla* luciferase gene). See Table 8.

Example 2

Synthetic *Renilla* Luciferase Nucleic Acid Molecule

The synthetic *Renilla* luciferase genes prepared include 1) an introduced Kozak sequence, 2) codon usage optimized for mammalian (human) expression, 3) a reduction or elimination of unwanted restriction sites, 4) removal of prokaryotic regulatory sites (ribosome binding site and TATA box), 5) removal of splice sites and poly(A) sites, and 6) a reduction or elimination of mammalian transcriptional factor binding sequences.

The process of computer-assisted design of synthetic *Renilla* luciferase genes by iterative rounds of codon optimization and removal of transcription factor binding sites and other regulatory sites as well as restriction sites can be described in three steps:

1. Using the wild type *Renilla* luciferase gene as the parent gene, codon usage was optimized, one amino acid was changed (T→A) to generate a Kozak consensus sequence, and undesired restriction sites were eliminated thereby creating synthetic gene Rlucver1.
2. Remove prokaryotic regulatory sites, splice sites, poly(A) sites and transcription factor (TF) binding sites (first pass). Then remove newly created TF binding sites. Then remove newly created undesired restriction enzyme sites, prokaryotic regulatory sites, splice sites, and poly(A) sites without introducing new TF binding sites. This thereby created Rlucver2.
3. Change 3 bases of Rlucver2 thereby creating Rluc-final.
4. The actual gene was then constructed from synthetic oligonucleotides corresponding to the Rluc-final designed sequence. All mutations resulting from the assembly or PCR process were corrected. This gene is Rluc-final.

Codon Selection

Starting with the *Renilla reniformis* luciferase sequence in Genbank (Accession No. M63501), codons were selected based on codon usage for optimal expression in human cells and to avoid *E. coli* low-usage codons. The best codon for expression in human cells (or the best two codons if found at a similar frequency) was chosen for all amino acids with more than one codon (Wada et al., 1990):

Arg: CGC	Lys: AAG
Leu: CTG	Asn: AAC
Ser: TCT/AGC	Gln: CAG
Thr: ACC	His: CAC
Pro: CCA/CCT	Glu: GAG
Ala: GCC	Asp: GAC
Gly: GGC	Tyr: TAC
Val: GTG	Cys: TGC
Ile: ATC/ATT	Phe: TTC

In cases where two codons were selected for one amino acid, they were used in an alternating fashion. To meet other criteria for the synthetic gene, the initial optimal codon selection was modified to some extent later. For example, introduction of a Kozak sequence required the use of GCT for Ala at amino acid position 2 (see below).

The following low-usage codons in mammalian cells were not used unless needed: Arg: CGA, CGU; Leu: CTA, UUA; Ser: TCG; Pro: CCG; Val: GTA; and Ile: ATA. The following low-usage codons in *E. coli* were also avoided when reasonable (note that 3 of these match the low-usage list for mammalian cells): Arg: CGA/CGG/AGA/AGG, Leu: CTA; Pro: CCC; Ile: ATA.

Introduction of Kozak Sequences

The Kozak sequence: 5′ aaccATGGCT 3′ (SEQ ID NO: 63) (the *Nco* I site is underlined, the coding region is shown in capital letters) was introduced to the synthetic *Renilla* luciferase gene. The introduction of the Kozak sequence changes the second amino acid from Thr to Ala (GCT).

Removal of undesired restriction sites

REBASE ver. 808 (updated August 1, 1998; Restriction Enzyme Database; www.neb.com/rebase) was employed to identify undesirable restriction sites as described in Example 1. The following undesired restriction sites (in addition to those described in Example 1) were removed according to the process described in Example 1: *Eco*ICR I, *Nde*I, *Nsi*I, *Sph*I, *Spe*I, *Xma*I, *Pst*I.

The version of *Renilla* luciferase (Rluc) which incorporates all these changes is Rlucver1.

Removal of prokaryotic (*E. coli*) regulatory sequences, splice sites, and poly(A) sites

The priority and process for eliminating transcription regulation sites was as described in Example 1.

Removal of TF binding sites

The same process, tools, and criteria were used as described in Example 1, however, the newer version 3.3 of the TRANSFAC database was employed.

After removing prokaryotic regulatory sequences, splice sites and poly(A) sites from Rlucver1, the first search for TF binding sites identified about 60 hits. All sites were eliminated with the exception of three that could not be removed without altering the amino acid sequence of the synthetic *Renilla* gene:

1. site at position 63 composed of two codons for W (TGGTGG), for CAC-binding protein T00076;
2. site at position 522 composed of codons for KMV (AAN ATG GTN), for myc-DF1 T00517;
3. site at position 885 composed of codons for EMG (GAR ATG GGN), for myc-DF1 T00517.

The subsequent second search for (newly introduced) TF binding sites yielded about 20 hits. All new sites were eliminated, leaving only the three sites described above. Finally, any newly introduced restriction sites, prokaryotic regulatory sequences, splice sites and poly(A) sites were removed without introducing new TF binding sites if possible.

Rlucver2 was obtained.

As in Example 1, lower stringency search parameters were specified for the TESS filtered string search to further evaluate the synthetic *Renilla* gene.

With the LLH reduced from 10 to 9 and the minimum element length reduced from 5 to 4, the TESS filtered string search did not show any new hits. When, in addition to the parameter changes listed above, the organism classification was expanded from "mammalia" to "chordata", the search yielded only four more TF binding sites. When the Min LLH was further reduced to between 8 and 0, the search showed two additional 5-base sites (MAMAG and CTKTK) which combined had four matches in Rlucver2, as well as several 4-base sites. Also as in Example 1, Rlucver2 was checked for hits to entries in the

EPD (Eukaryotic Promoter Database, Release 45). Three hits were determined one to Mus musculus promoter H-2L^d (Cell, 44, 261 (1986)), one to Herpes Simplex Virus type 1 promoter b'g'2.7 kb, and one to Homo sapiens DHFR promoter (J. Mol. Biol., 176, 169 (1984)). However, no further changes were made to Rlucver2.

Summary of Properties for Rlucver2

- All 30 low usage codons were eliminated. The introduction of a Kozak sequence changed the second amino acid from Thr to Ala;
- base composition: 55.7% GC (*Renilla* wild-type parent gene: 36.5%);
- one undesired restriction site could not be eliminated: *Eco*R V at position 488;
- the synthetic gene had no prokaryotic promoter sequence but one potentially functional ribosome binding site (RBS) at positions 867-73 (about 13 bases upstream of a Met codon) could not be eliminated;
- all poly(A) sites were eliminated;
- splice sites: 2 donor splice sites could not be eliminated (both share the amino acid sequence MGK);
- TF sites: all sites with a consensus of >4 unambiguous bases were eliminated (about 280 TF binding sites were removed) with 3 exceptions due to the preference to avoid changes to the amino acid sequence.

When introduced into pGL3, Rluc-final has a Kozak sequence (CACCATGGCT; SEQ ID NO:65). The changes in Rluc-final relative to Rlucver2 were introduced during gene assembly. One change was at position 619, a C to an A, which eliminated a eukaryotic promoter sequence and reduced the stability of a hairpin structure in the corresponding oligonucleotide employed to assemble the gene. Other changes included a change from CGC to AGA at positions 218-220 (resulted in a better oligonucleotide for PCR).

Gene Assembly Strategy

The gene assembly protocol employed for the synthetic *Renilla* luciferase was similar to that described in Example 1.

Sense Strand primer:

5′ AACCATGGCTTCCAAGGTGTACGACCCCGAGCAACGCAAA 3′ (SEQ ID NO:66)

Anti-sense Strand primer:

5′ GCTCTAGAATTACTGCTCGTTCTTCAGCACGCGCTCCACG 3′ (SEQ ID NO:67)

The resulting synthetic gene fragment was cloned into a pRAM vector using *Nco* I and *Xba* I. Two clones having the correct size insert were sequenced. Four to six mutations were found in the synthetic gene from each clone. These mutations were fixed by site-directed mutagenesis (Gene Editor from Promega Corp., Madison, WI) and swapping the correct regions between these two genes. The corrected gene was confirmed by sequencing.

Other Vectors

To prepare an expression vector for the synthetic *Renilla* luciferase gene in a pGL-3 control vector backbone, 5 μg of pGL3-control was digested with *Nco* I and *Xba* I in 50 μl final volume with 2 μl of each enzyme and 5 μl 10X buffer B (nanopure water was used to fill the volume to 50 μl). The digestion reaction was incubated at 37°C for 2 hours, and the whole mixture was run on a 1% agarose gel in 1XTAE. The desired vector backbone fragment was purified using Qiagen's QIAquick gel extraction kit.

The native *Renilla* luciferase gene fragment was cloned into pGL3-control vector using two oligonucleotides, *Nco* I-RL-F and *Xba* I-RL-R, to PCR amplify native *Renilla* luciferase gene using pRL-CMV as the template. The sequence for *Nco* I-RL-F is 5′-CGCTAGCCATGGCTTCGAAAGTTTATGATCC -3′ (SEQ ID NO:68); the sequence for *Xba* I-RL-R is 5′ GGCCAGTAACTCTAGAATTATTGTT-3′ (SEQ ID NO:69). The PCR reaction was carried out as follows:

Reaction mixture (for 100 μl):

DNA template (Plasmid)	1.0 μl (1.0 ng/μl final)
10 X Rec. Buffer	10.0 μl (Stratagene Corp.)

dNTPs (25 mM each)	1.0 µl (final 250 µM)
Primer 1 (10 µM)	2.0 µl (0.2 µM final)
Primer 2 (10 µM)	2.0 µl (0.2 µM final)
Pfu DNA Polymerase	2.0 µl (2.5 U/µl, Stratagene Corp.)
	82.0 µl double distilled water

PCR Reaction: heat 94°C for 2 minutes; (94°C for 20 seconds; 65°C for 1 minute; 72°C for 2 minutes; then 72°C for 5 minutes) x 25 cycles, then incubate on ice. The PCR amplified fragment was cut from a gel, and the DNA purified and stored at –20°C.

To introduce native *Renilla* luciferase gene fragment into pGL3-control vector, 5 µg of the PCR product of the native *Renilla* luciferase gene (RAM-RL-synthetic) was digested with *Nco* I and *Xba* I. The desired *Renilla* luciferase gene fragment was purified and stored at -20°C.

Then 100 ng of insert and 100 ng of pGL3-control vector backbone were digested with restriction enzymes *Nco* I and *Xba* I and ligated together. Then 2 µl of the ligation mixture was transformed into JM109 competent cells. Eight ampicillin resistance clones were picked and their DNA isolated. DNA from each positive clone of pGL3-control-native and pGL3-control-synthetic was purified. The correct sequences for the native gene and the synthetic gene in the vectors were confirmed by DNA sequencing.

To determine whether the synthetic *Renilla* luciferase gene has improved expression in mammalian cells, the gene was cloned into the mammalian expression vector pGL3-control vector under the control of SV40 promoter and SV40 early enhancer. The native *Renilla* luciferase gene was also cloned into the pGL-3 control vector so that the expression from synthetic gene and the native gene could be compared. The expression vectors were then transfected into four common mammalian cell lines (CHO, NIH3T3, Hela and CV-1; Table 9), and the expression levels compared between the vectors with the synthetic gene versus the native gene. The amount of DNA used was at two different levels to ascertain that expression from the synthetic gene is consistently

increased at different expression levels. The results show a 70-600 fold increase of expression for the synthetic *Renilla* luciferase gene in these cells (Table 4).

Table 4

Cell Type	Amount Vector	Fold Expression Increase
CHO	0.2 μg	142
	2.8 μg	145
NIH3T3	0.2 μg	326
	2.0 μg	593
HeLa	0.2 μg	185
	1.0 μg	103
CV-1	0.2 μg	68
	2.0 μg	72

One important advantage of luciferase reporter is its short protein half-life. The enhanced expression could also result from extended protein half-life and, if so, this gives an undesired disadvantage of the new gene. This possibility is ruled out by a cycloheximide chase ("CHX Chase") experiment, which demonstrated that there was no increase of protein half-life resulted from the humanized *Renilla* luciferase gene.

To ensure that the increase in expression is not limited to one expression vector backbone, is promoter specific and/or cell specific, a synthetic *Renilla* gene (Rluc-final) as well as native *Renilla* gene were cloned into different vector backbones and under different promoters. The synthetic gene always exhibited increased expression compared to its wild-type counterpart (Table 5).

Table 5

Vector	NIH-3T3	HeLa	CHO
pRL-tk, native	3,834.6	922.4	7,671.9
pRL-tk, synthetic	13,252.5	9,040.2	41,743.5
pRL-CMV, native	168,062.2	842,482.5	153,539.5
pRL-CMV, synthetic	2,168,129	8,440,306	2,532,576
pRL-SV40, native	224,224.4	346,787.6	85,323.6

Vector	NIH-3T3	HeLa	CHO
pRL-SV40, synthetic	1,469,588	2,632,510	1,422,830
pRL-null, native	2,853.8	431.7	2,434
pRL-null, synthetic	9,151.17	2,439	28,317.1
pRGL3b, native	12	21.8	17
pRGL3b, synthetic	130.5	212.4	1,094.5
pRGL3-tk, native	27.9	155.5	186.4
pRGL3-tk, synthetic	6,778.2	8,782.5	9,685.9
pRL-tk no intron, native	31.8	165	93.4
pRL-tk no intron, synthetic	6,665.5	6,379	21,433.1

Table 6

	Percent of control vector		
Vector	CHO cells	NIH3T3 cells	HeLa cells
pRL-control native	100	100	100
pRL-control synthetic	100	100	100
pRL-basic native	4.1	5.6	0.2
pRL-basic synthetic	0.4	0.1	0.0
pRL-promoter native	5.9	7.8	0.6
pRL-promoter synthetic	15.0	9.9	1.1
pRL-enhancer native	42.1	123.9	52.7
pRL-enhancer synthetic	2.6	1.5	5.4

With reduced spurious expression the synthetic gene should exhibit less basal level transcription in a promoterless vector. The synthetic and native *Renilla* luciferase genes were cloned into the pGL3-basic vector to compare the basal level of transcription. Because the synthetic gene itself has increased expression efficiency, the activity from the promoterless vector cannot be compared directly to judge the difference in basal transcription, rather, this is taken into consideration by comparing the percentage of activity from the

promoterless vector in reference to the control vector (expression from the basic vector divided by the expression in the fully functional expression vector with both promoter and enhancer elements). The data demonstrate that the synthetic *Renilla* luciferase has a lower level of basal transcription than the native gene in mammalian cells (Table 6).

It is well known to those skilled in the art that an enhancer can substantially stimulate promoter activity. To test whether the synthetic gene has reduced risk of inappropriate transcriptional characteristics, the native and synthetic gene were introduced into a vector with an enhancer element (pGL3-enhancer vector). Because the synthetic gene has higher expression efficiency, the activity of both cannot be compared directly to compare the level of transcription in the presence of the enhancer, however, this is taken into account by using the percentage of activity from enhancer vector in reference to the control vector (expression in the presence of enhancer divided by the expression in the fully functional expression vector with both promoter and enhancer elements). Such results show that when native gene is present, the enhancer alone is able to stimulate transcription from 42-124% of the control, however, when the native gene is replaced by the synthetic gene in the same vector, the activity only constitutes 1-5% of the value when the same enhancer and a strong SV40 promoter are employed. This clearly demonstrates that synthetic gene has reduced risk of spurious expression (Table 6).

The synthetic *Renilla* gene (Rluc-final) was used in *in vitro* systems to compare translation efficiency with the native gene. In a T7 quick coupled transcription/translation system (Promega Corp., Madison, WI), pRL-null native plasmid (having the native *Renilla* luciferase gene under the control of the T7 promoter) or the same amount of pRL-null-synthetic plasmid (having the synthetic *Renilla* luciferase gene under the control of the T7 promoter) was added to the TNT reaction mixture and luciferase activity measured every 5 minutes up to 60 minutes. Dual Luciferase assay kit (Promega Corp.) was used to measure *Renilla* luciferase activity. The data showed that improved expression was obtained from the synthetic gene. To further evidence the increased translation efficiency of the synthetic gene, RNA was prepared by an *in vitro* transcription system, then purified. pRL-null (native or synthetic) vectors

were linearized with *Bam*HI. The DNA was purified by multiple phenol-chloroform extraction followed by ethanol precipitation. An *in vitro* T7 transcription system was employed by prepare RNAs. The DNA template was removed by using RNase-free DNase, and RNA was purified by phenol-chloroform extraction followed by multiple isopropanol precipitations. The same amount of purified RNA, either for the synthetic gene or the native gene, was then added to a rabbit reticulocyte lysate or wheat germ lysate. Again, the synthetic *Renilla* luciferase gene RNA produced more luciferase than the native one. These data suggest that the translation efficiency is improved by the synthetic sequence. To determine why the synthetic gene was highly expressed in wheat germ, plant codon usage was determined. The lowest usage codons in higher plants coincided with those in mammals.

Reporter gene assays are widely used to study transcriptional regulation events. This is often carried out in co-transfection experiments, in which, along with the primary reporter construct containing the testing promoter, a second control reporter under a constitutive promoter is transfected into cells as an internal control to normalize experimental variations including transfection efficiencies between the samples. Control reporter signal, potential promoter cross talk between the control reporter and primary reporter, as well as potential regulation of the control reporter by experimental conditions, are important aspects to consider for selecting a reliable co-reporter vector.

As described above, vector constructs were made by cloning synthetic *Renilla* luciferase gene into different vector backbones under different promoters. All the constructs showed higher expression in the three mammalian cell lines tested (Table 5). Thus, with better expression efficiency, the synthetic *Renilla* luciferase gives out higher signal when transfected into mammalian cells.

Because a higher signal is obtained, less promoter activity is required to achieve the same reporter signal, this reduced risk of promoter interference. CHO cells were transfected with 50 ng pGL3-control (firefly *luc+)* plus one of 5 different amounts of native pRL-TK plasmid (50, 100, 500, 1000, or 2000 ng) or synthetic pRL-TK (5, 10, 50, 100, or 200 ng). To each transfection, pUC19 carrier DNA was added to a total of 3 μg DNA. 10 fold less pRL-TK DNA gave

similar or more signal as the native gene, with reduced risk of inhibiting expression from the primary reporter pGL3-control.

Experimental treatment sometimes may activate cryptic sites within the gene and cause induction or suppression of the co-reporter expression, which would compromise its function as co-reporter for normalization of transfection efficiencies. One example is that TPA induces expression of co-reporter vectors harboring the wild-type gene when transfecting MCF-7 cells. 500 ng pRL-TK (native), 5 μg native and synthetic pRG-B, 2.5 μg native and synthetic pRG-TK were transfected per well of MCF-7 cells. 100 ng/well pGL3-control (firefly luc+) was co-transfected with all RL plasmids. Carrier DNA, pUC19, was used to bring the total DNA transfected to 5.1 μg/well. 15.3 μl TransFast Transfection Reagent (Promega Corp., Madison, WI) was added per well. Sixteen hours later, cells were trypsinized, pooled and split into six wells of a 6-well dish and allowed to attach to the well for 8 hours. Three wells were then treated with the 0.2 nM of the tumor promoter, TPA (phorbol-12-myristate-13-acetate, Calbiochem #524400-S), and three wells were mock treated with 20 μl DMSO. Cells were harvested with 0.4 ml Passive Lysis Buffer 24 hours post TPA addition. The results showed that by using the synthetic gene, undesirable change of co-reporter expression by experimental stimuli can be avoided (Table 7). This demonstrates that using synthetic gene can reduce the risk of anomalous expression.

Table 7

Vector	Rlu	Fold Induction
pRL-tk untreated (native)	184	
pRL-tk TPA treated (native)	812	4.4
pRG-B untreated (native)	1	
pRG-B TPA treated (native)	8	8.0
pRG-B untreated (final)	132	
pRG-B TPA treated (final)	195	1.47
pRG-tk untreated (native)	44	

Vector	Rlu	Fold Induction
pRG-tk TPA treated (native)	192	4.36
pRG-tk untreated (final)	12,816	
pRG-tk TPA treated (final)	11,347	0.88

Example 3

Synthetic Firefly Luciferase Genes

The *luc*+ gene (U.S. Patent No. 5,670,356) was optimized using two approaches. In the first approach (Strategy A), regulatory sequences such as codons were optimized and consensus transcription factor binding sites (TFBS) were removed (see Example 4, although different versions of programs and databases were used). The sequences obtained for the first approach include hluc+ver2AF1 through hluc+ver2AF8 (designations with an "F" indicate the construct included flanking sequences). hluc+ver2AF1 is codon-optimized, hluc+ver2AF2 is a sequence obtained after a first round of removal of identified undesired sequences including transcription factor binding sites, hluc+ver2AF3 was obtained after a second round of removal of identified undesired sequences including transcription factor binding sites, hluc+ver2AF4 was obtained after a third round of removal of identified undesired sequences including transcription factor binding sites, hluc+ver2AF5 was obtained after a fourth round of removal of identified undesired sequences including transcription factor binding sites, hluc+ver2AF6 was obtained after removal of promoter modules and RBS, hluc+ver2AF7 was obtained after further removal of identified undesired sequences including transcription factor binding sites, and hluc+ver2AF8 was obtained after modifying a restriction enzyme recognition site.

Pairwise DNA identity of different *P.pyralis* luciferase gene versions:

Table 8

	luc	luc+	hluc+	hluc+ver2A1	hluc+ver2B1	hluc+ver2A6	hluc+ver2B6
Luc	100	95	76	73	77	74	75
luc+		100	78	76	78	75	77
hluc+			100	91	81	87	81
hluc+ver2A1				100	74	91	78
hluc+ver2B1					100	74	85
hluc+ver2A6						100	80
hluc+ver2B6							100

luc+ has the following sequence:

atggaagacgccaaaaacataaagaaaggcccggcgccattctatccgctggaagatggaaccgctggagagca
actgcataaggctatgaagagatacgccctggttcctggaacaattgcttttacagatgcacatatcgaggtggacatc
acttacgctgagtacttcgaaatgtccgttcggttggcagaagctatgaaacgatatgggctgaatacaaatcacaga
atcgtcgtatgcagtgaaaactctcttcaattctttatgccggtgttgggcgcgttatttatcggagttgcagttgcgccc
gcgaacgacatttataatgaacgtgaattgctcaacagtatgggcatttcgcagcctaccgtggtgttcgtttccaaaa
aggggttgcaaaaaattttgaacgtgcaaaaaaagctcccaatcatccaaaaaattattatcatggattctaaaacgga
ttaccagggatttcagtcgatgtacacgttcgtcacatctcatctacctcccggttttaatgaatacgattttgtgccaga
gtccttcgatagggacaagacaattgcactgatcatgaactcctctggatctactggtctgcctaaaggtgtcgctctg
cctcatagaactgcctgcgtgagattctcgcatgccagagatcctatttttggcaatcaaatcattccggatactgcgat
tttaagtgttgttccattccatcacggttttggaatgtttactacactcggatatttgatatgtggatttcgagtcgtcttaat
gtatagatttgaagaagagctgtttctgaggagccttcaggattacaagattcaaagtgcgctgctggtgccaaccccta
ttctccttcttcgccaaaagcactctgattgacaaatacgatttatctaatttacacgaaattgcttctggtggcgctcccc
tctctaaggaagtcggggaagcggttgccaagaggttccatctgccaggtatcaggcaaggatatgggctcactga
gactacatcagctattctgattacacccgagggggatgataaaccgggcgcggtcggtaaagttgttccatttttgaa
gcgaaggttgtggatctggataccgggaaaacgctgggcgttaatcaaagaggcgaactgtgtgtgagaggtccta
tgattatgtccggttatgtaaacaatccggaagcgaccaacgccttgattgacaaggatggatggctacattctggag
acatagcttactgggacgaagacgaacacttcttcatcgttgaccgcctgaagtctctgattaagtacaaaggctatca
ggtggctcccgctgaattggaatccatcttgctccaacaccccaacatcttcgacgcaggtgtcgcaggtcttcccga
cgatgacgccggtgaacttcccgccgccgttgttgttttggagcacggaaagacgatgacggaaaaagagatcgtg
gattacgtcgccagtcaagtaacaaccgcgaaaaagttgcgcggaggagttgtgtttgtggacgaagtaccgaaag
gtcttaccggaaaactcgacgcaagaaaaatcagagagatcctcataaggccaagaagggcggaaagatcgcc
gtgtaa (SEQ ID NO:43)

and hluc+ has the following sequence:

atggccgatgctaagaacattaagaagggccctgctcccttctaccctctggaggatggcaccgctggcgagcagc
tgcacaaggccatgaagaggtatgccctggtgcctggcaccattgccttcaccgatgcccacattgaggtggacatc
acctatgccgagtacttcgagatgtctgtgcgcctggccgaggccatgaagaggtacggcctgaacaccaaccacc
gcatcgtggtgtgctctgagaactctctgcagttcttcatgccagtgctgggcgccctgttcatcggagtggccgtgg
cccctgctaacgacatttacaacgagcgcgagctgctgaacagcatgggcatttctcagcctaccgtggtgttcgtgt
ctaagaagggcctgcagaagatcctgaacgtgcagaagaagctgcctatcatccagaagatcatcatcatggactct
aagaccgactaccagggcttccagagcatgtacacattcgtgacatctcatctgcctcctggcttcaacgagtacgac

ttcgtgccagagtctttcgacagggacaaaaccattgccctgatcatgaacagctctgggtctaccggcctgcctaag
ggcgtggccctgcctcatcgcaccgcctgtgtgcgcttctctcacgcccgcgaccctattttcggcaaccagatcatc
cccgacaccgctattctgagcgtggtgccattccaccacggcttcggcatgttcaccaccctgggctacctgatttgc
ggctttcgggtggtgctgatgtaccgcttcgaggaggagctgttcctgcgcagcctgcaagactacaaaattcagtct
gccctgctggtgccaaccctgttcagcttcttcgctaagagcaccctgatcgacaagtacgacctgtctaacctgcac
gagattgcctctggcggcgccccactgtctaaggaggtgggcgaagccgtggccaagcgctttcatctgccaggca
tccgccagggctacggcctgaccgagacaaccagcgccattctgattaccccagagggcgacgacaagcctggc
gccgtgggcaaggtggtgccattcttcgaggccaaggtggtggacctggacaccggcaagaccctgggagtgaa
ccagcgcggcgagctgtgtgtgcgcggccctatgattatgtccggctacgtgaataaccctgaggccacaaacgcc
ctgatcgacaaggacggctggctgcactctggcgacattgcctactgggacgaggacgagcacttcttcatcgtgga
ccgcctgaagtctctgatcaagtacaagggctaccaggtggccccagccgagctggagtctatcctgctgcagcac
cctaacattttcgacgccggagtggccggcctgcccgacgacgatgccggcgagctgcctgccgccgtcgtcgtg
ctggaacacggcaagaccatgaccgagaaggagatcgtggactatgtggccagccaggtgacaaccgccaagaa
gctgcgcggcggagtggtgttcgtggacgaggtgcccaagggcctgaccggcaagctggacgcccgcaagatcc
gcgagatcctgatcaaggctaagaaaggcggcaagatcgccgtgtaa (SEQ ID NO:14).

Table 9

Percent Identity

		hluc+ver2A8	hluc+ver2B10	luc+	hluc+
	hluc+ver2A8		79.6	74	86.6
Divergence	hluc+ver2B10	22.9		75.9	80.1
	luc+	30.4	27.8		77.4
	hluc+	14.7	22.5	25.7	

Table 10

Composition statistics of different *P.pyralis* luciferase gene versions

	GC content	CG di-nucleotides
H. sapiens	53%	--
luc	45%	99
luc+	47%	97
hluc+	60%	111
hluc+ver2A1	66%	151
hluc+ver2B1	46%	1
hluc+ver2A6	58%	133
hluc+ver2B6	49%	53

hluc+ver2A1-hluc+ver2A5 have the following sequences (SEQ ID Nos.16-20):

hluc+ver2A1

AAAGCCACCATGGAGGACGCCAAGAACATCAAGAAGGGCCCCGCCC
CCTTCTACCCCCTGGAGGACGGCACCGCCGGCGAGCAGCTGCACAAG
GCCATGAAGCGCTACGCCCTGGTGCCCGGCACCATCGCCTTCACCGA
CGCCCACATCGAGGTGGACATCACCTACGCCGAGTACTTCGAGATGA
GCGTGCGCCTGGCCGAGGCCATGAAGCGCTACGGCCTGAACACCAAC
CACCGCATCGTGGTGTGCAGCGAGAACAGCCTGCAGTTCTTCATGCC
CGTGCTGGGCGCCCTGTTCATCGGCGTGGCCGTGGCCCCCGCCAACG
ACATCTACAACGAGCGCGAGCTGCTGAACAGCATGGGCATCAGCCAG
CCCACCGTGGTGTTCGTGAGCAAGAAGGGCCTGCAGAAGATCCTGAA
CGTGCAGAAGAAGCTGCCCATCATCCAGAAGATCATCATCATGGACA
GCAAGACCGACTACCAGGGCTTCCAGAGCATGTACACCTTCGTGACC
AGCCACCTGCCCCCCGGCTTCAACGAGTACGACTTCGTGCCCGAGAG
CTTCGACCGCGACAAGACCATCGCCCTGATCATGAACAGCAGCGGCA
GCACCGGCCTGCCCAAGGGCGTGGCCCTGCCCCACCGCACCGCCTGC
GTGCGCTTCAGCCACGCCCGCGACCCCATCTTCGGCAACCAGATCAT
CCCCGACACCGCCATCCTGAGCGTGGTGCCCTTCCACCACGGCTTCG
GCATGTTCACCACCCTGGGCTACCTGATCTGCGGCTTCCGCGTGGTGC
TGATGTACCGCTTCGAGGAGGAGCTGTTCCTGCGCAGCCTGCAGGAC
TACAAGATCCAGAGCGCCCTGCTGGTGCCCACCCTGTTCAGCTTCTTC
GCCAAGAGCACCCTGATCGACAAGTACGACCTGAGCAACCTGCACGA
GATCGCCAGCGGCGGCGCCCCCCTGAGCAAGGAGGTGGGCGAGGCC
GTGGCCAAGCGCTTCCACCTGCCCGGCATCCGCCAGGGCTACGGCCT
GACCGAGACCACCAGCGCCATCCTGATCACCCCCGAGGGCGACGACA
AGCCCGGCGCCGTGGGCAAGGTGGTGCCCTTCTTCGAGGCCAAGGTG
GTGGACCTGGACACCGGCAAGACCCTGGGCGTGAACCAGCGCGGCG
AGCTGTGCGTGCGCGGCCCCATGATCATGAGCGGCTACGTGAACAAC
CCCGAGGCCACCAACGCCCTGATCGACAAGGACGGCTGGCTGCACAG
CGGCGACATCGCCTACTGGGACGAGGACGAGCACTTCTTCATCGTGG
ACCGCCTGAAGAGCCTGATCAAGTACAAGGGCTACCAGGTGGCCCCC
GCCGAGCTGGAGAGCATCCTGCTGCAGCACCCCAACATCTTCGACGC

CGGCGTGGCCGGCCTGCCCGACGACGACGCCGGCGAGCTGCCCGCCG
CCGTGGTGGTGCTGGAGCACGGCAAGACCATGACCGAGAAGGAGAT
CGTGGACTACGTGGCCAGCCAGGTGACCACCGCCAAGAAGCTGCGCG
GCGGCGTGGTGTTCGTGGACGAGGTGCCCAAGGGCCTGACCGGCAAG
CTGGACGCCCGCAAGATCCGCGAGATCCTGATCAAGGCCAAGAAGG
GCGGCAAGATCGCCGTGTAATAATTCTAGA

hluc+ver2A2

AAAGCCACCATGGAGGACGCCAAGAACATCAAGAAGGGCCCAGCGC
CATTCTACCCCCTGGAGGACGGCACCGCCGGCGAGCAGCTGCACAAG
GCCATGAAGCGCTACGCCCTGGTGCCCGGCACCATCGCCTTCACCGA
CGCACATATCGAGGTGGACATCACCTACGCCGAGTACTTCGAGATGA
GCGTTCGGCTGGCAGAGGCTATGAAGCGCTATGGGCTGAACACCAAC
CATCGCATCGTGGTGTGCAGCGAGAACAGCTTGCAGTTCTTCATGCC
CGTGTTGGGTGCCCTGTTCATCGGCGTGGCTGTGGCCCCAGCTAACG
ACATCTACAACGAGCGCGAGCTGCTGAACAGCATGGGCATCAGCCAG
CCCACCGTCGTATTCGTGAGCAAGAAAGGGCTGCAAAAGATCCTGAA
CGTGCAAAAGAAGCTGCCCATCATCCAAAAGATCATCATCATGGACA
GCAAGACCGACTACCAGGGCTTCCAAAGCATGTACACCTTCGTGACC
AGCCATTTGCCGCCCGGCTTCAACGAGTACGACTTCGTGCCCGAGAG
CTTCGACCGCGACAAGACCATCGCCCTGATCATGAACAGTAGTGGCA
GTACCGGCTTACCTAAGGGCGTGGCCCTACCGCACCGCACCGCCTGT
GTCCGATTCAGTCATGCCCGCGACCCCATCTTCGGCAACCAGATCATC
CCCGACACCGCTATCCTGAGCGTGGTGCCATTTCACCACGGCTTCGGC
ATGTTCACCACCCTGGGCTACTTGATCTGCGGCTTCCGGGTCGTGCTG
ATGTACCGCTTCGAGGAGGAGCTATTCTTGCGCAGCTTGCAAGACTA
CAAGATTCAAAGCGCCCTGCTGGTGCCCACCCTGTTCAGTTTCTTCGC
CAAGAGCACCCTGATCGACAAGTACGACCTGAGCAACCTGCACGAG
ATCGCCAGCGGCGGCGCCCCGCTCAGCAAGGAGGTGGGCGAGGCCG
TGGCCAAGCGCTTCCACCTGCCAGGCATCCGCCAGGGCTACGGCCTG
ACCGAGACAACCAGCGCCATTCTGATCACCCCCGAGGGGGACGACA
AGCCTGGCGCAGTAGGCAAGGTGGTGCCCTTCTTCGAGGCTAAGGTG
GTGGACCTGGACACCGGTAAAACCCTGGGTGTGAACCAGCGCGGCG

AGCTGTGCGTCCGTGGCCCCATGATCATGAGCGGCTACGTTAACAAC
CCCGAGGCTACAAACGCCCTGATCGACAAGGACGGCTGGCTGCACAG
CGGCGACATCGCCTACTGGGACGAGGACGAGCACTTCTTCATCGTGG
ACCGGCTGAAGAGCCTGATCAAATACAAGGGCTACCAGGTAGCCCCA
GCCGAACTGGAGAGCATCCTGCTGCAGCACCCCAACATCTTCGACGC
CGGGGTCGCCGGCCTGCCCGACGACGATGCCGGCGAGCTGCCCGCCG
CAGTCGTGGTGCTGGAGCACGGTAAAACCATGACCGAGAAGGAGAT
CGTGGACTATGTGGCCAGCCAGGTTACAACCGCCAAGAAGCTGCGCG
GCGGCGTGGTGTTCGTGGACGAGGTGCCTAAAGGCCTGACGGGCAAG
TTGGACGCCCGCAAGATCCGCGAGATTCTGATCAAGGCCAAGAAGGG
CGGCAAGATCGCCGTGTAATAATTCTAGA

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AAAGCCACCATGGAAGATGCCAAAAACATTAAGAAGGGCCCAGCGC
CATTCTACCCACTGGAGGACGGCACCGCCGGCGAGCAGCTGCACAAA
GCCATGAAGCGCTACGCCCTGGTGCCCGGCACCATCGCCTTTACCGA
CGCACATATCGAGGTGGACATCACCTACGCCGAGTACTTCGAGATGA
GCGTTCGGCTGGCAGAGGCTATGAAGCGCTATGGGCTGAATACCAAC
CATCGCATCGTGGTGTGCAGCGAGAATAGCTTGCAGTTCTTCATGCCC
GTGTTGGGTGCCCTGTTCATCGGTGTGGCTGTGGCCCCAGCTAACGAC
ATCTACAACGAGCGCGAGCTGCTGAACAGCATGGGCATCAGCCAGCC
CACCGTCGTATTCGTGAGCAAGAAAGGGCTGCAAAAGATCCTCAACG
TGCAAAAGAAGCTACCGATCATACAAAAGATCATCATCATGGATAGC
AAGACCGACTACCAGGGCTTCCAAAGCATGTACACCTTCGTGACCAG
CCATTTGCCACCCGGCTTCAACGAGTACGACTTCGTGCCCGAGAGCTT
CGACCGGGACAAAACCATCGCCCTGATCATGAACAGTAGTGGCAGTA
CCGGATTGCCCAAGGGCGTAGCCCTACCGCACCGCACCGCCTGTGTC
CGATTCAGTCATGCCCGCGACCCCATCTTCGGCAACCAGATCATCCCC
GACACCGCTATCCTCAGCGTGGTGCCATTTCACCACGGCTTCGGCATG
TTCACCACGCTGGGCTACTTGATCTGCGGCTTTCGGGTCGTGCTCATG
TACCGCTTCGAGGAGGAGCTATTCTTGCGCAGCTTGCAAGACTATAA
GATTCAAAGCGCCCTGCTGGTGCCCACACTGTTCAGCTTCTTCGCCAA
GAGCACTCTCATCGACAAGTACGACCTGAGCAACCTGCACGAGATCG

CCAGCGGCGGGGCGCCGCTCAGCAAGGAGGTGGGCGAGGCCGTGGC
CAAGCGCTTCCACCTACCAGGCATCCGCCAGGGCTACGGCCTGACAG
AAACAACCAGCGCCATTCTGATCACCCCCGAAGGGGACGACAAGCCT
GGCGCAGTAGGCAAGGTGGTGCCCTTCTTCGAGGCTAAGGTGGTGGA
CTTGGACACCGGTAAGACCCTGGGTGTGAACCAGCGCGGCGAGCTGT
GCGTCCGTGGCCCCATGATCATGAGCGGCTACGTTAACAACCCCGAG
GCTACAAACGCTCTCATCGACAAGGACGGCTGGCTGCACAGCGGCGA
CATCGCCTACTGGGACGAGGACGAGCACTTCTTCATCGTGGACCGGC
TGAAGAGCCTGATCAAATACAAGGGCTACCAGGTAGCCCCAGCCGA
ACTGGAGAGCATCCTGCTGCAACACCCCAACATCTTCGACGCCGGGG
TCGCCGGCCTGCCCGACGACGATGCCGGCGAGCTGCCCGCCGCAGTC
GTCGTGCTGGAGCACGGTAAAACCATGACCGAGAAGGAGATCGTGG
ACTATGTGGCCAGCCAGGTTACAACCGCCAAGAAGCTGCGCGGTGGT
GTTGTGTTCGTGGACGAGGTGCCTAAAGGCCTGACGGGCAAGTTGGA
CGCCCGCAAGATCCGCGAGATTCTCATTAAGGCCAAGAAGGGCGGCA
AGATCGCCGTGTAATAATTCTAGA

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AAAGCCACCATGGAAGATGCCAAAAACATTAAGAAGGGCCCAGCGC
CATTCTACCCACTCGAAGACGGCACCGCCGGCGAGCAGCTGCACAAA
GCCATGAAGCGCTACGCCCTGGTGCCCGGCACCATCGCCTTTACCGA
CGCACATATCGAGGTGGACATTACCTACGCCGAGTACTTCGAGATGA
GCGTTCGGCTGGCAGAAGCTATGAAGCGCTATGGGCTGAACACCAAC
CATCGCATCGTGGTGTGCAGCGAGAATAGCTTGCAGTTCTTCATGCCC
GTGTTGGGTGCCCTGTTCATCGGTGTGGCTGTGGCCCCAGCTAACGAC
ATCTACAACGAGCGCGAGCTGCTGAACAGCATGGGCATCAGCCAGCC
CACCGTCGTATTCGTGAGCAAGAAAGGGCTGCAAAAGATCCTCAACG
TGCAAAAGAAGCTACCGATCATACAAAAGATCATCATCATGGATAGC
AAGACCGACTACCAGGGCTTCCAAAGCATGTACACCTTCGTGACTTC
CCATTTGCCACCCGGCTTCAACGAGTACGACTTCGTGCCCGAGAGCTT
CGACCGGGACAAAACCATCGCCCTGATCATGAACAGTAGTGGCAGTA
CCGGATTGCCCAAGGGCGTAGCCCTACCGCACCGCACCGCTTGTGTC
CGATTCAGTCATGCCCGCGACCCCATCTTCGGCAACCAGATCATCCCC

GACACCGCTATCCTCAGCGTGGTGCCATTTCACCACGGCTTCGGCATG
TTCACCACGCTGGGCTACTTGATCTGCGGCTTTCGGGTCGTGCTCATG
TACCGCTTCGAGGAGGAGCTATTCTTGCGCAGCTTGCAAGACTATAA
GATTCAAAGCGCCCTGCTGGTGCCCACACTGTTCAGTTTCTTCGCCAA
GAGCACTCTCATCGACAAGTACGACCTAAGCAACTTGCACGAGATCG
CCAGCGGCGGGGCGCCGCTCAGCAAGGAGGTGGGCGAGGCCGTGGC
CAAACGCTTCCACCTACCAGGCATCCGCCAGGGCTACGGCCTGACAG
AAACAACCAGCGCCATTCTGATCACCCCCGAAGGGGACGACAAGCCT
GGCGCAGTAGGCAAGGTGGTGCCCTTCTTCGAGGCTAAGGTGGTGGA
CTTGGACACCGGTAAGACACTGGGTGTGAACCAGCGCGGCGAGCTGT
GCGTCCGTGGCCCCATGATCATGAGCGGCTACGTTAACAACCCCGAG
GCTACAAACGCTCTCATCGACAAGGACGGCTGGCTGCACAGCGGCGA
CATCGCCTACTGGGACGAGGACGAGCACTTCTTCATCGTGGACCGGC
TGAAGAGCCTGATCAAATACAAGGGCTACCAGGTAGCCCCAGCCGA
ACTGGAGAGCATCCTGCTGCAACACCCCAACATCTTCGACGCCGGGG
TCGCCGGCCTGCCCGACGACGATGCCGGCGAGCTGCCCGCCGCAGTC
GTCGTGCTGGAACACGGTAAAACCATGACCGAGAAGGAGATCGTGG
ACTATGTGGCCAGCCAGGTTACAACCGCCAAGAAGCTGCGCGGTGGT
GTTGTGTTCGTGGACGAGGTGCCTAAAGGCCTGACGGGCAAGTTGGA
CGCCCGCAAGATCCGCGAGATTCTCATTAAGGCCAAGAAGGGCGGCA
AGATCGCCGTGTAATAATTCTAGA

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AAAGCCACCATGGAAGATGCCAAAAACATTAAGAAGGGCCCAGCGC
CATTCTACCCACTCGAAGACGGCACCGCCGGCGAGCAGCTGCACAAA
GCCATGAAGCGCTACGCCCTGGTGCCCGGCACCATCGCCTTTACCGA
CGCACATATCGAGGTGGACATTACCTACGCCGAGTACTTCGAGATGA
GCGTTCGGCTGGCAGAAGCTATGAAGCGCTATGGGCTGAACACCAAC
CATCGGATCGTGGTGTGCAGCGAGAATAGCTTGCAGTTCTTCATGCC
CGTGTTGGGTGCCCTGTTCATCGGTGTGGCTGTGGCCCCAGCTAACGA
CATCTACAACGAGCGCGAGCTGCTGAACAGCATGGGCATCAGCCAGC
CCACCGTCGTATTCGTGAGCAAGAAAGGGCTGCAAAAGATCCTCAAC
GTGCAAAAGAAGCTACCGATCATACAAAAGATCATCATCATGGATAG

CAAGACCGACTACCAGGGCTTCCAAAGCATGTACACCTTCGTGACTT
CCCATTTGCCACCCGGCTTCAACGAGTACGACTTCGTGCCCGAGAGC
TTCGACCGGGACAAAACCATCGCCCTGATCATGAACAGTAGTGGCAG
TACCGGATTGCCCAAGGGCGTAGCCCTACCGCACCGCACCGCTTGTG
TCCGATTCAGTCATGCCCGCGACCCCATCTTCGGCAACCAGATCATCC
CCGACACCGCTATCCTCAGCGTGGTGCCATTTCACCACGGCTTCGGCA
TGTTCACCACGCTGGGCTACTTGATCTGCGGCTTTCGGGTCGTGCTCA
TGTACCGCTTCGAGGAGGAGCTATTCTTGCGCAGCTTGCAAGACTAT
AAGATTCAAAGCGCCCTGCTGGTGCCCACACTGTTCAGTTTCTTCGCT
AAGAGCACTCTCATCGACAAGTACGACCTAAGCAACTTGCACGAGAT
CGCCAGCGGCGGGGCGCCGCTCAGCAAGGAGGTGGGCGAGGCCGTG
GCCAAACGCTTCCACCTACCAGGCATCCGCCAGGGCTACGGCCTGAC
AGAAACAACCAGCGCCATTCTGATCACCCCCGAAGGGGACGACAAG
CCTGGCGCAGTAGGCAAGGTGGTGCCCTTCTTCGAGGCTAAGGTGGT
GGACTTGGACACCGGTAAGACACTGGGTGTGAACCAGCGCGGCGAG
CTGTGCGTCCGTGGCCCCATGATCATGAGCGGCTACGTTAACAACCC
CGAGGCTACAAACGCTCTCATCGACAAGGACGGCTGGCTGCACAGCG
GCGACATCGCCTACTGGGACGAGGACGAGCACTTCTTCATCGTGGAC
CGGCTGAAGAGCCTGATCAAATACAAGGGCTACCAGGTAGCCCCAGC
CGAACTGGAGAGCATCCTGCTGCAACACCCCAACATCTTCGACGCCG
GGGTCGCCGGCCTGCCCGACGACGATGCCGGCGAGCTGCCCGCCGCA
GTCGTCGTGCTGGAACACGGTAAAACCATGACCGAGAAGGAGATCGT
GGACTATGTGGCCAGCCAGGTTACAACCGCCAAGAAGCTGCGCGGTG
GTGTTGTGTTCGTGGACGAGGTGCCTAAAGGCCTGACGGGCAAGTTG
GACGCCCGCAAGATCCGCGAGATTCTCATTAAGGCCAAGAAGGGCG
GCAAGATCGCCGTGTAATAATTCTAGA

hluc+ver2A6 has the following sequence

AAAGCCACCATGGAaGAtGCCAAaAACATtAAGAAGGGCCCaGCgCCaT
TCTACCCaCTcGAaGACGGCACCGCCGGCGAGCAGCTGCACAAaGCCA
TGAAGCGCTACGCCCTGGTGCCCGGCACCATCGCCTTtACCGACGCaC
AtATCGAGGTGGACATtACCTACGCCGAGTACTTCGAGATGAGCGTtCG
gCTGGCaGAaGCtATGAAGCGCTAtGGgCTGAAtACaAACCAtCGgATCGT

GGTGTGCAGCGAGAAtAGCtTGCAGTTCTTCATGCCCGTGtTGGGtGCC
CTGTTCATCGGtGTGGCtGTGGCCCCaGCtAACGACATCTACAACGAGC
GCGAGCTGCTGAACAGCATGGGCATCAGCCAGCCCACCGTcGTaTTCG
TGAGCAAGAAaGGgCTGCAaAAGATCCTcAACGTGCAaAAGAAGCTaCC
gATCATaCAaAAGATCATCATCATGGAtAGCAAGACCGACTACCAGGG
CTTCCAaAGCATGTACACCTTCGTGACttcCCAttTGCCaCCCGGCTTCAA
CGAGTACGACTTCGTGCCCGAGAGCTTCGACCGgGACAAaACCATCGC
CCTGATCATGAACAGtAGtGGCAGtACCGGatTgCCcAAGGGCGTaGCCC
TaCCgCACCGCACCGCtTGtGTcCGaTTCAGtCAtGCCCGCGACCCCATCT
TCGGCAACCAGATCATCCCCGACACCGCtATCCTcAGCGTGGTGCCaTT
tCACCACGGCTTCGGCATGTTCACCACgCTGGGCTACtTGATCTGCGGC
TTtCGgGTcGTGCTcATGTACCGCTTCGAGGAGGAGCTaTTCtTGCGCAG
CtTGCAaGACTAtAAGATtCAaAGCGCCCTGCTGGTGCCCACaCTGTTCA
GtTTCTTCGCtAAGAGCACtCTcATCGACAAGTACGACCTaAGCAACtTG
CACGAGATCGCCAGCGGCGGgGCgCCgCTcAGCAAGGAGGTaGGtGAG
GCCGTGGCCAAaCGCTTCCACCTaCCaGGCATCCGCCAGGGCTACGGC
CTGACaGAaACaACCAGCGCCATtCTGATCACCCCCGAaGGgGACGACA
AGCCtGGCGCaGTaGGCAAGGTGGTGCCCTTCTTCGAGGCtAAGGTGGT
GGACtTGGACACCGGtAAgACaCTGGGtGTGAACCAGCGCGGCGAGCTG
TGCGTcCGtGGCCCCATGATCATGAGCGGCTACGTtAACAACCCCGAG
GCtACaAACGCtCTcATCGACAAGGACGGCTGGCTGCACAGCGGCGAC
ATCGCCTACTGGGACGAGGACGAGCACTTCTTCATCGTGGACCGgCT
GAAGAGCCTGATCAAaTACAAGGGCTACCAGGTaGCCCCaGCCGAaCT
GGAGAGCATCCTGCTGCAaCACCCCAACATCTTCGACGCCGGgGTcGC
CGGCCTGCCCGACGACGAtGCCGGCGAGCTGCCCGCCGCaGTcGTcGT
GCTGGAaCACGGtAAaACCATGACCGAGAAGGAGATCGTGGACTAtGT
GGCCAGCCAGGTtACaACCGCCAAGAAGCTGCGCGGtGGtGTtGTGTTC
GTGGACGAGGTGCCtAAaGGCCTGACgGGCAAGtTGGACGCCCGCAAG
ATCCGCGAGATtCTcATtAAGGCCAAGAAGGGCGGCAAGATCGCCGTG
TAATAATTCTAGA (SEQ ID NO:21).

The hluc+ver2A6 sequence was modified yielding hluc+ver2A7:

AAAGCCACCATGGAaGAtGCCAAaAACATtAAGAA
GGGCCCaGCgCCaTTCTACCCaCTcGAaGACGGgACCGCCGGCGAGCAG
CTGCACAAaGCCATGAAGCGCTACGCCCTGGTGCCCGGCACCATCGC
CTTtACCGACGCaCAtATCGAGGTGGACATtACCTACGCCGAGTACTTC
GAGATGAGCGTtCGgCTGGCaGAaGCtATGAAGCGCTAtGGgCTGAAtAC
aAACCAtCGgATCGTGGTGTGCAGCGAGAAtAGCtTGCAGTTCTTCATGC
CCGTGtTGGGtGCCCTGTTCATCGGtGTGGCtGTGGCCCCaGCtAACGAC
ATCTACAACGAGCGCGAGCTGCTGAACAGCATGGGCATCAGCCAGCC
CACCGTcGTaTTCGTGAGCAAGAAaGGgCTGCAaAAGATCCTcAACGTG
CAaAAGAAGCTaCCgATCATaCAaAAGATCATCATCATGGAtAGCAAGA
CCGACTACCAGGGCTTCCAaAGCATGTACACCTTCGTGACttcCCAttTG
CCaCCCGGCTTCAACGAGTACGACTTCGTGCCCGAGAGCTTCGACCGg
GACAAaACCATCGCCCTGATCATGAACAGtAGtGGCAGtACCGGatTgCC
cAAGGGCGTaGCCCTaCCgCACCGCACCGCtTGtGTcCGaTTCAGtCAtGCC
CGCGACCCCATCTTCGGCAACCAGATCATCCCCGACACCGCtATCCTc
AGCGTGGTGCCaTTtCACCACGGCTTCGGCATGTTCACCACgCTGGGCT
ACtTGATCTGCGGCTTtCGgGTcGTGCTcATGTACCGCTTCGAGGAGGAG
CTaTTCtTGCGCAGCtTGCAaGACTAtAAGATTcAatctGCCCTGCTGGTGC
CCACaCTaTTtAGcTTCTTCGCtAAGAGCACtCTcATCGACAAGTACGACC
TaAGCAACtTGCACGAGATCGCCAGCGGCGGgGCgCCgCTcAGCAAGGA
GGTaGGtGAGGCCGTGGCCAAaCGCTTCCACCTaCCaGGCATCCGCCAG
GGCTACGGCCTGACaGAaACaACCAGCGCCATtCTGATCACCCCCGAaG
GgGACGACAAGCCtGGCGCaGTaGGCAAGGTGGTGCCCTTCTTCGAGG
CtAAGGTGGTGGACtTGGACACCGGtAAgACaCTGGGtGTGAACCAGCG
CGGCGAGCTGTGCGTcCGtGGCCCCATGATCATGAGCGGCTACGTtAA
CAACCCCGAGGCtACaAACGCtCTcATCGACAAGGACGGCTGGCTGCA
CAGCGGCGACATCGCCTACTGGGACGAGGACGAGCACTTCTTCATCG
TGGACCGgCTGAAGAGCCTGATCAAaTACAAGGGCTACCAGGTaGCCC
CaGCCGAaCTGGAGAGCATCCTGCTGCAaCACCCCAACATCTTCGACG
CCGGgGTcGCCGGCCTGCCCGACGACGAtGCCGGCGAGCTGCCCGCCG
CaGTcGTcGTGCTGGAaCACGGtAAaACCATGACCGAGAAGGAGATCGT
GGACTAtGTGGCCAGCCAGGTtACaACCGCCAAGAAGCTGCGCGGtGGt
GTtGTGTTCGTGGACGAGGTGCCtAAaGGCCTGACgGGCAAGtTGGACG

CCCGCAAGATCCGCGAGATtCTcATtAAGGCCAAGAAGGGCGGCAAGA
TCGCCGTGTAATAATTCTAGA (SEQ ID NO:22).

For vectors with a *BglI* site in the multiple cloning region, the *BglI* site present in the firefly sequence can be removed. The luciferase gene from hluc+ver2AF8, which lacks a *BglI* site, displays an average of a 7.2-fold increase in expression when assayed in four mammalian cell lines, i.e., NIH3T3, CHO, HeLa and HEK293 cells.

hluc+ver2A8 has the following sequence:
AAAGCCACCATGGAaGAtGCCAAaAACATtAAGAAGGGCCCaGCgCCaT
TCTACCCaCTcGAaGACGGgACCGCCGGCGAGCAGCTGCACAAaGCCA
TGAAGCGCTACGCCCTGGTGCCCGGCACCATCGCCTTtACCGACGCaC
AtATCGAGGTGGACATtACCTACGCCGAGTACTTCGAGATGAGCGTtCG
gCTGGCaGAaGCtATGAAGCGCTAtGGgCTGAAtACaAACCAtCGgATCGT
GGTGTGCAGCGAGAAtAGCtTGCAGTTCTTCATGCCCGTGtTGGGtGCC
CTGTTCATCGGtGTGGCtGTGGCCCCaGCtAACGACATCTACAACGAGC
GCGAGCTGCTGAACAGCATGGGCATCAGCCAGCCCACCGTcGTaTTCG
TGAGCAAGAAaGGgCTGCAaAAGATCCTcAACGTGCAaAAGAAGCTaCC
gATCATaCAaAAGATCATCATCATGGAtAGCAAGACCGACTACCAGGG
CTTCCAaAGCATGTACACCTTCGTGACttcCCAttTGCCaCCCGGCTTCAA
CGAGTACGACTTCGTGCCCGAGAGCTTCGACCGgGACAAaACCATCGC
CCTGATCATGAACAGtAGtGGCAGtACCGGatTgCCcAAGGGCGTaGCCC
TaCCgCACCGCACCGCtTGtGTcCGaTTCAGtCAtGCCCGCGACCCCATCT
TCGGCAACCAGATCATCCCCGACACCGCtATCCTcAGCGTGGTGCCaTT
tCACCACGGCTTCGGCATGTTCACCACgCTGGGCTACtTGATCTGCGGC
TTtCGgGTcGTGCTcATGTACCGCTTCGAGGAGGAGCTaTTCtTGCGCAG
CtTGCAaGACTAtAAGATtCAatctGCCCTGCTGGTGCCCACaCTaTTtAGcT
TCTTCGCtAAGAGCACtCTcATCGACAAGTACGACCTaAGCAACtTGCAC
GAGATCGCCAGCGGCGGgGCgCCgCTcAGCAAGGAGGTaGGtGAGGCC
GTGGCCAAaCGCTTCCACCTaCCaGGCATCCGCCAGGGCTACGGCCTG
ACaGAaACaACCAGCGCCATtCTGATCACCCCCGAaGGgGACGACAAGC
CtGGCGCaGTaGGCAAGGTGGTGCCCTTCTTCGAGGCtAAGGTGGTGGA
CtTGGACACCGGtAAgACaCTGGGtGTGAACCAGCGCGGCGAGCTGTGC

GTcCGtGGCCCCATGATCATGAGCGGCTACGTtAACAACCCCGAGGCtA
CaAACGCtCTcATCGACAAGGACGGCTGGCTGCACAGCGGCGACATCG
CCTACTGGGACGAGGACGAGCACTTCTTCATCGTGGACCGgCTGAAG
AGCCTGATCAAaTACAAGGGCTACCAGGTaGCCCCaGCCGAaCTGGAG
AGCATCCTGCTGCAaCACCCCAACATCTTCGACGCCGGgGTcGCCGGC
CTGCCCGACGACGAtGCCGGCGAGCTGCCCGCCGCaGTcGTcGTGCTGG
AaCACGGtAAaACCATGACCGAGAAGGAGATCGTGGACTAtGTGGCCA
GCCAGGTtACaACCGCCAAGAAGCTGCGCGGtGGtGTtGTGTTCGTGGA
CGAGGTGCCtAAaGGaCTGACcGGCAAGtTGGACGCCCGCAAGATCCGC
GAGATtCTcATtAAGGCCAAGAAGGGCGGCAAGATCGCCGTGTAATAA
TTCTAGA (SEQ ID NO:23).

For the second approach, firefly luciferase *luc*+ codons were optimized for mammalian expression, and the number of consensus transcription factor binding site, and CG dinucleotides (CG islands, potential methylation sites) was reduced. The second approach yielded: versions hluc+ver2BF1 through hluc+ver2BF5. hluc+ver2BF1 is codon-optimized, hluc+ver2BF2 is a sequence obtained after a first round of removal of identified undesired sequences including transcription factor binding sites, hluc+ver2BF3 was obtained after a second round of removal of identified undesired sequences including transcription factor binding sites, hluc+ver2BF4 was obtained after a third round of removal of identified undesired sequences including transcription factor binding sites, hluc+ver2BF5 was obtained after a fourth round of removal of identified undesired sequences including transcription factor binding sites, hluc+ver2BF6 was obtained after removal of promoter modules and RBS, hluc+ver2BF7 was obtained after further removal of identified undesired sequences including transcription factor binding sites, and hluc+ver2BF8 was obtained after modifying a restriction enzyme recognition site.

hluc+ver2B1-B5 have the following sequences (SEQ ID Nos. 24-28):

hluc+ver2B1

AAAGCCACCATGGAGGATGCTAAGAATATTAAGAAGGGGCCTGCTCC
TTTTTATCCTCTGGAGGATGGGACAGCTGGGGAGCAGCTGCATAAGG

CTATGAAGAGATATGCTCTGGTGCCTGGGACAATTGCTTTTACAGATG
CTCATATTGAGGTGGATATTACATATGCTGAGTATTTTGAGATGTCTG
TGAGACTGGCTGAGGCTATGAAGAGATATGGGCTGAATACAAATCAT
AGAATTGTGGTGTGTTCTGAGAATTCTCTGCAGTTTTTTATGCCTGTG
CTGGGGGCTCTGTTTATTGGGGTGGCTGTGGCTCCTGCTAATGATATT
TATAATGAGAGAGAGCTGCTGAATTCTATGGGGATTTCTCAGCCTAC
AGTGGTGTTTGTGTCTAAGAAGGGGCTGCAGAAGATTCTGAATGTGC
AGAAGAAGCTGCCTATTATTCAGAAGATTATTATTATGGATTCTAAG
ACAGATTATCAGGGGTTTCAGTCTATGTATACATTTGTGACATCTCAT
CTGCCTCCTGGGTTTAATGAGTATGATTTTGTGCCTGAGTCTTTTGAT
AGAGATAAGACAATTGCTCTGATTATGAATTCTTCTGGGTCTACAGG
GCTGCCTAAGGGGGTGGCTCTGCCTCATAGAACAGCTTGTGTGAGAT
TTTCTCATGCTAGAGATCCTATTTTTGGGAATCAGATTATTCCTGATA
CAGCTATTCTGTCTGTGGTGCCTTTTCATCATGGGTTTGGGATGTTTAC
AACACTGGGGTATCTGATTTGTGGGTTTAGAGTGGTGCTGATGTATAG
ATTTGAGGAGGAGCTGTTTCTGAGATCTCTGCAGGATTATAAGATTCA
GTCTGCTCTGCTGGTGCCTACACTGTTTTCTTTTTTTGCTAAGTCTACA
CTGATTGATAAGTATGATCTGTCTAATCTGCATGAGATTGCTTCTGGG
GGGGCTCCTCTGTCTAAGGAGGTGGGGGAGGCTGTGGCTAAGAGATT
TCATCTGCCTGGGATTAGACAGGGGTATGGGCTGACAGAGACAACAT
CTGCTATTCTGATTACACCTGAGGGGGATGATAAGCCTGGGGCTGTG
GGGAAGGTGGTGCCTTTTTTTGAGGCTAAGGTGGTGGATCTGGATAC
AGGGAAGACACTGGGGGTGAATCAGAGAGGGGAGCTGTGTGTGAGA
GGGCCTATGATTATGTCTGGGTATGTGAATAATCCTGAGGCTACAAA
TGCTCTGATTGATAAGGATGGGTGGCTGCATTCTGGGGATATTGCTTA
TTGGGATGAGGATGAGCATTTTTTTATTGTGGATAGACTGAAGTCTCT
GATTAAGTATAAGGGGTATCAGGTGGCTCCTGCTGAGCTGGAGTCTA
TTCTGCTGCAGCATCCTAATATTTTTGATGCTGGGGTGGCTGGGCTGC
CTGATGATGATGCTGGGGAGCTGCCTGCTGCTGTGGTGGTGCTGGAG
CATGGGAAGACAATGACAGAGAAGGAGATTGTGGATTATGTGGCTTC
TCAGGTGACAACAGCTAAGAAGCTGAGAGGGGGGGTGGTGTTTGTGG
ATGAGGTGCCTAAGGGGCTGACAGGGAAGCTGGATGCTAGAAAGAT
TAGAGAGATTCTGATTAAGGCTAAGAAGGGGGGGAAGATTGCTGTGT

AATAATTCTAGA

hluc+ver2B2

AAAGCCACCATGGAAGATGCTAAAAACATTAAGAAGGGGCCTGCTCC
TTTCTACCCTCTGGAGGATGGGACTGCCGGGGAGCAGCTGCATAAAG
CTATGAAGCGGTATGCTCTGGTGCCAGGCACAATTGCGTTCACGGAT
GCTCACATTGAGGTGGACATTACATACGCTGAGTATTTTGAGATGTCG
GTGCGGCTGGCTGAGGCTATGAAGCGATATGGGCTGAATACAAACCA
TAGAATTGTAGTGTGCTCTGAGAACTCGTTGCAGTTTTTTATGCCTGT
GCTGGGGGCTCTCTTCATCGGGGTGGCTGTGGCTCCTGCTAACGACAT
TTACAATGAGAGAGAGCTTTTGAACTCGATGGGGATTTCTCAGCCTA
CAGTGGTGTTTGTGAGTAAGAAAGGGCTTCAAAAGATTCTCAATGTG
CAAAAGAAGCTGCCTATTATTCAAAAGATTATTATTATGGACTCTAA
GACAGACTACCAGGGGTTTCAGTCTATGTATACATTTGTGACATCTCA
TCTGCCTCCTGGGTTCAACGAGTATGACTTTGTGCCCGAGTCTTTCGA
CAGAGATAAGACAATTGCTCTGATTATGAATTCATCTGGGTCTACCG
GGCTGCCTAAGGGTGTAGCTCTGCCACATAGAACAGCTTGTGTGAGA
TTTTCTCATGCTAGGGACCCTATTTTTGGGAATCAGATTATTCCTGAT
ACTGCTATTCTGTCGGTTGTGCCCTTTCATCATGGGTTTGGGATGTTTA
CAACACTGGGCTACCTGATATGTGGGTTTAGAGTGGTGCTCATGTATA
GGTTTGAGGAGGAGCTTTTTTTGCGCTCTCTGCAAGATTATAAGATTC
AGTCTGCTCTGCTGGTGCCTACACTGTTTTCTTTTTTTGCTAAGTCTAC
CCTGATCGATAAGTATGATCTGTCCAACCTGCACGAGATTGCTTCTGG
GGGGGCTCCTCTGTCTAAGGAGGTAGGTGAGGCTGTGGCTAAGCGCT
TTCATCTGCCTGGAATCAGACAGGGGTATGGGCTAACAGAAACAACA
TCTGCTATTCTGATTACACCAGAGGGGGATGATAAGCCCGGGGCTGT
AGGGAAAGTGGTGCCCTTTTTTGAAGCTAAAGTAGTTGATCTTGATAC
CGGTAAGACACTGGGGGTGAATCAGCGAGGGGAACTGTGTGTGAGA
GGGCCTATGATTATGTCGGGGTATGTGAACAACCCTGAGGCTACAAA
TGCTCTGATTGATAAGGATGGGTGGCTGCATTCGGGCGATATTGCTTA
CTGGGATGAGGATGAGCATTTCTTCATCGTGGACAGACTGAAGTCGT
TGATCAAATATAAGGGGTATCAAGTAGCTCCTGCTGAGCTGGAGTCC
ATTCTGCTTCAACATCCTAACATTTTCGATGCTGGGGTGGCTGGGCTG

CCTGATGATGATGCTGGGGAGCTGCCTGCTGCTGTAGTGGTGCTGGA
GCACGGTAAGACAATGACAGAGAAGGAGATTGTGGATTATGTGGCTT
CACAAGTGACAACAGCTAAGAAACTGAGAGGTGGCGTTGTGTTTGTG
GATGAGGTGCCTAAAGGGCTGACAGGCAAGCTGGATGCTAGAAAAA
TTCGAGAGATTCTGATTAAGGCTAAGAAGGGTGGAAAGATTGCTGTG
TAATAGTTCTAGA

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AAAGCCACCATGGAAGATGCTAAAAACATTAAGAAGGGGCCTGCTCC
TTTCTACCCTCTTGAAGATGGGACTGCTGGCGAGCAACTTCACAAAG
CTATGAAGCGGTATGCTCTTGTGCCAGGCACAATTGCGTTCACGGAT
GCTCACATTGAGGTGGACATCACATACGCTGAGTATTTTGAGATGTC
GGTGCGGCTGGCAGAAGCTATGAAGCGCTATGGGCTGAATACAAACC
ATAGAATTGTAGTGTGCAGTGAGAACTCGTTGCAGTTCTTTATGCCCG
TGCTGGGGGCTCTCTTCATCGGGGTGGCTGTGGCTCCTGCTAACGACA
TCTACAACGAGCGAGAGCTGTTGAACTCGATGGGGATTTCTCAGCCT
ACAGTGGTGTTTGTGAGTAAGAAAGGGCTTCAAAAGATTCTCAATGT
GCAAAAGAAGCTGCCTATTATTCAAAAGATTATTATTATGGACTCTA
AGACCGACTACCAGGGGTTTCAGTCTATGTATACATTTGTGACATCTC
ATCTGCCTCCTGGCTTCAACGAGTACGACTTCGTGCCCGAGTCTTTCG
ACAGAGATAAGACAATTGCTCTGATCATGAATTCATCCGGGTCTACC
GGGCTGCCTAAGGGTGTAGCTCTGCCCCATAGAACAGCTTGTGTGAG
ATTTTCTCATGCTAGGGACCCTATTTTTGGGAATCAGATTATTCCTGA
CACTGCTATTCTGTCGGTGGTGCCCTTTCATCATGGGTTTGGGATGTT
TACAACACTGGGCTACCTAATATGTGGGTTTAGAGTGGTGCTCATGTA
TAGGTTTGAAGAAGAGCTGTTCTTACGCTCTTTGCAAGATTATAAGAT
TCAGTCTGCTCTGCTGGTGCCAACACTATTCTCTTTTTTTGCTAAGTCT
ACGCTCATAGACAAGTATGACTTGTCCAACTTGCACGAGATTGCTTCT
GGCGGAGCACCTCTGTCTAAGGAGGTAGGTGAGGCTGTGGCTAAGCG
CTTTCATCTGCCTGGTATCAGACAGGGGTATGGGCTAACAGAAACAA
CATCTGCTATTCTGATTACACCAGAGGGGGATGATAAGCCCGGGGCT
GTAGGGAAAGTGGTGCCCTTTTTTGAAGCCAAAGTAGTTGATCTTGAT
ACCGGTAAGACACTAGGGGTGAACCAGCGTGGTGAACTGTGTGTGAG

AGGGCCTATGATTATGTCGGGGTACGTTAACAACCCCGAAGCTACAA
ATGCTCTGATTGATAAGGATGGCTGGCTGCATTCGGGCGACATTGCTT
ACTGGGATGAGGATGAGCATTTCTTCATCGTGGACAGACTGAAGTCG
TTGATCAAATACAAGGGGTATCAAGTAGCTCCTGCTGAGCTGGAATC
CATTCTGCTTCAACATCCCAACATTTTCGATGCTGGGGTGGCTGGGCT
GCCTGATGATGATGCTGGGGAGTTGCCTGCTGCTGTAGTGGTGCTTGA
GCACGGTAAGACAATGACAGAGAAGGAGATCGTGGATTATGTGGCTT
CACAAGTGACAACAGCTAAGAAACTGAGAGGTGGCGTTGTGTTTGTG
GATGAGGTGCCTAAAGGGCTCACTGGCAAGCTGGATGCTAGAAAAAT
TCGAGAGATTCTGATTAAGGCTAAGAAGGGTGGAAAGATTGCTGTGT
AATAGTTCTAGA

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AAAGCCACCATGGAAGATGCTAAAAACATTAAGAAGGGGCCTGCTCC
CTTCTACCCTCTTGAAGATGGGACTGCTGGCGAGCAACTTCACAAAG
CTATGAAGCGGTATGCTCTTGTGCCAGGCACAATTGCGTTCACGGAT
GCTCACATTGAGGTGGACATCACATACGCTGAGTATTTTGAGATGTC
GGTGCGGCTGGCAGAAGCTATGAAGCGCTATGGGCTGAATACAAACC
ATAGAATTGTAGTGTGCAGTGAGAACTCGTTGCAGTTCTTTATGCCCG
TGCTGGGGGCTCTCTTCATCGGGGTGGCTGTGGCTCCTGCTAACGACA
TCTACAACGAGCGAGAGCTGTTGAACTCGATGGGGATCTCTCAGCCT
ACAGTGGTGTTTGTGAGTAAGAAAGGGCTTCAAAAGATTCTCAATGT
GCAAAAGAAGCTGCCTATTATTCAAAAGATTATTATTATGGACTCTA
AGACAGACTACCAGGGGTTTCAGTCCATGTATACATTTGTGACATCTC
ATCTGCCTCCTGGCTTCAACGAGTACGACTTCGTGCCCGAGTCTTTCG
ACAGAGATAAGACAATTGCTCTGATCATGAATTCATCCGGGTCTACC
GGGCTGCCTAAGGGTGTAGCTCTGCCCCATCGAACAGCTTGTGTGAG
ATTCTCTCATGCCAGGGACCCGATCTTTGGGAATCAGATTATTCCTGA
CACTGCTATTCTGTCGGTGGTGCCCTTTCATCATGGGTTTGGGATGTT
TACAACACTGGGATACCTAATATGTGGGTTTAGAGTGGTGCTCATGT
ATAGGTTTGAAGAAGAACTGTTCTTACGCTCTTTGCAAGATTATAAGA
TTCAGTCTGCTCTGCTGGTGCCAACACTATTCTCTTTTTTTGCTAAGTC
TACGCTCATAGACAAGTATGACTTGTCCAACTTGCACGAGATTGCTTC

TGGCGGAGCACCTCTGTCTAAGGAGGTAGGTGAGGCTGTGGCTAAGC
GCTTTCATCTGCCTGGTATCAGACAGGGGTACGGGCTAACAGAAACA
ACTTCTGCTATTCTGATTACACCAGAGGGCGATGACAAGCCCGGGGC
TGTAGGGAAAGTGGTGCCCTTTTTTGAAGCCAAAGTAGTTGATCTTGA
TACCGGTAAGACACTAGGGGTGAACCAGCGTGGTGAACTGTGTGTGC
GGGGCCCTATGATTATGTCGGGGTACGTTAACAACCCCGAAGCTACA
AATGCTCTTATTGATAAGGATGGCTGGTTGCATTCGGGCGACATTGCC
TACTGGGATGAGGATGAGCATTTCTTCATCGTGGACAGACTGAAGTC
GTTGATCAAATACAAGGGGTATCAAGTAGCTCCTGCTGAGCTGGAAT
CCATTCTGCTTCAACATCCAAACATTTTCGATGCTGGGGTGGCTGGGC
TGCCTGATGATGATGCTGGAGAGTTGCCTGCTGCTGTAGTAGTGCTTG
AGCACGGTAAGACAATGACAGAGAAGGAGATCGTGGATTATGTGGC
TTCACAAGTGACAACAGCTAAGAAACTGAGAGGTGGCGTTGTGTTTG
TGGATGAGGTGCCTAAAGGGCTCACTGGCAAGCTGGATGCCAGAAAA
ATTCGAGAGATTCTCATTAAGGCTAAGAAGGGTGGAAAGATTGCTGT
GTAATAGTTCTAGA

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AAAGCCACCATGGAAGATGCTAAAAACATTAAGAAGGGGCCTGCTCC
CTTCTACCCTCTTGAAGATGGGACTGCTGGCGAGCAACTTCACAAAG
CTATGAAGCGGTATGCTCTTGTGCCAGGCACAATTGCGTTCACGGAT
GCTCACATTGAGGTGGACATCACATACGCTGAGTATTTTGAGATGTC
GGTGCGGCTGGCAGAAGCTATGAAGCGCTATGGGCTGAATACAAACC
ATAGAATTGTAGTGTGCAGTGAGAACTCGTTGCAGTTCTTTATGCCCG
TGCTGGGGGCTCTCTTCATCGGGGTGGCTGTGGCTCCTGCTAACGACA
TCTACAACGAGCGAGAGCTGTTGAACTCGATGGGGATCTCTCAGCCT
ACAGTGGTGTTTGTGAGTAAGAAAGGGCTTCAAAAGATTCTCAATGT
GCAAAAGAAGCTGCCTATTATACAAAAGATTATTATTATGGACTCTA
AGACCGACTACCAGGGGTTTCAGTCCATGTACACATTTGTAACCTCTC
ATCTGCCTCCTGGCTTCAACGAGTACGACTTCGTGCCCGAGTCTTTCG
ACAGGGACAAAACGATTGCTCTGATCATGAACTCATCCGGGTCTACC
GGGCTGCCTAAGGGTGTAGCTCTGCCCCATCGAACAGCTTGTGTGAG
ATTCTCTCATGCCAGGGACCCGATCTTTGGGAATCAGATTATTCCTGA

CACTGCTATTCTGTCGGTGGTGCCCTTTCATCATGGGTTTGGGATGTT
CACAACACTGGGATACCTCATTTGCGGGTTTAGAGTGGTGCTCATGTA
TAGGTTTGAAGAAGAACTATTCCTACGCTCTTTGCAAGATTATAAGAT
TCAGTCTGCTCTGCTGGTGCCAACACTATTCTCTTTTTTTGCTAAGTCT
ACGCTCATAGACAAGTATGACTTGTCCAACTTGCACGAGATTGCTTCT
GGCGGAGCACCTCTGTCTAAGGAGGTAGGTGAGGCTGTGGCTAAGCG
CTTTCATCTGCCTGGTATCAGACAGGGGTACGGGCTAACAGAAACAA
CTTCTGCTATTCTGATTACACCAGAGGGCGATGACAAACCCGGGGCT
GTAGGGAAAGTGGTGCCCTTTTTTGAAGCCAAAGTAGTTGATCTTGAT
ACCGGTAAGACACTAGGGGTGAACCAGCGTGGTGAACTGTGTGTGCG
GGGCCCTATGATTATGTCGGGGTACGTTAACAACCCCGAAGCTACAA
ATGCTCTTATTGATAAGGATGGCTGGTTGCATTCGGGCGACATTGCCT
ACTGGGATGAGGATGAGCATTTCTTCATCGTGGACAGACTGAAGTCG
TTGATCAAATACAAGGGGTATCAAGTAGCTCCTGCTGAGCTGGAATC
CATTCTGCTTCAACATCCTAACATTTTCGATGCTGGGGTGGCTGGGCT
GCCTGATGATGATGCTGGAGAGTTGCCTGCTGCTGTAGTAGTGCTTGA
GCACGGTAAGACAATGACAGAGAAGGAGATCGTGGATTATGTGGCTT
CACAAGTGACAACAGCTAAGAAACTGAGAGGTGGCGTTGTGTTTGTG
GATGAGGTGCCTAAAGGGCTCACTGGCAAGCTGGATGCCAGAAAAAT
TCGAGAGATTCTCATTAAGGCTAAGAAGGGTGGAAAGATTGCTGTGT
AATAGTTCTAGA

hluc+ver2B6 has the following sequence:

AAAGCCACCATGGAaGATGCcAAaAAcATTAAGAAGGGGCCTGCTCCc
TTcTAcCCTCTtGAaGATGGGACtGCtGGcGAGCAaCTtCAcAAaGCTATGA
AGcGgTATGCTCTtGTGCCaGGcACAATTGCgTTcACgGATGCTCAcATTG
AaGTaGAcATcACATAcGCTGAGTATTTTGAGATGTCgGTGcGgCTGGCa
GAaGCTATGAAGcGcTATGGGCTGAATACAAAcCATAGAATTGTaGTGT
GcagTGAGAAcTCgtTGCAGTTcTTTATGCCcGTGCTGGGGGCTCTcTTcAT
cGGGGTGGCTGTGGCTCCTGCTAAcGAcATcTAcAAcGAGcGAGAGCTgt
TGAAcTCgATGGGGATcTCTCAGCCTACAGTGGTGTTTGTGagTAAGAA
aGGGCTtCAaAAGATTCTcAATGTGCAaAAGAAGCTGCCTATTATaCAaA
AGATTATTATTATGGAcTCtAAGACcGAcTAcCAGGGGTTTCAGTCcATG

TAcACATTTGTaACcTCTCATCTGCCTCCTGGcTTcAAcGAGTAcGAcTTc
GTGCCcGAGTCTTTcGAcAGgGAcAAaACgATTGCTCTGATcATGAAcagc
TCcGGGTCTACcGGGCTGCCTAAGGGtGTaGCTCTGCCcCATcGAACAGC
TTGTGTGAGATTcTCTCATGCcAGgGAcCCgATcTTtGGaAAcCAGATcATc
CCTGAcACtGCTATTCTGTCgGTgGTGCCcTTTCATCATGGGTTTGGGAT
GTTcACAACACTGGGaTAccTcATtTGcGGGTTTAGAGTGGTGCTcATGTA
TAGgTTTGAaGAaGAaCTaTTccTacGcTCTtTGCAaGATTATAAGATTCAG
TCTGCTCTGCTGGTGCCaACACTaTTcTCTTTTTTTGCTAAGTCTACgCTc
ATaGAcAAGTATGActTGTCcAActTGCAcGAGATTGCTTCTGGcGGaGCa
CCTCTGTCTAAGGAGGTaGGtGAGGCTGTGGCTAAGcGcTTTCATCTGC
CTGGtATcAGACAGGGGTAcGGGCTaACAGAaACAACtTCTGCTATTCTG
ATTACACCaGAGGGcGATGAcAAaCCcGGGGCTGTaGGGAAaGTGGTGC
CcTTTTTTGAaGCcAAaGTaGTtGATCTtGATACcGGtAAGACACTaGGGGT
GAAcCAGcGtGGtGAaCTGTGTGTGcGgGGcCCTATGATTATGTCgGGGTA
cGTtAAcAAcCCcGAaGCTACAAATGCTCTcATaGAcAAGGAcGGgTGGcTt
CATagcGGcGAcATTGCcTAcTGGGAcGAGGATGAGCATTTcTTcATcGTG
GAcAGACTGAAGTCgtTGATcAAaTAcAAGGGGTATCAaGTaGCTCCTGC
TGAGCTGGAaTCcATTCTGCTtCAaCAcCCcAAtATcTTcGATGCTGGGGT
GGCTGGGCTGCCTGATGATGATGCTGGaGAGcTGCCTGCTGCTGTaGTa
GTGCTtGAGCAcGGtAAGACAATGACAGAGAAGGAGATcGTGGATTAT
GTGGCTTCaCAaGTGACAACAGCTAAGAAaCTGAGAGGtGGcGTtGTGT
TTGTGGATGAGGTGCCTAAaGGGCTcACtGGcAAGCTGGATGCcAGAAA
aATTcGAGAGATTCTcATTAAGGCTAAGAAGGGtGGaAAGATTGCTGTG
TAATAgTTCTAGA (SEQ ID NO:29).

hluc+ver2BF8 was created by removing a *Ptx*1 consensus transcription factor binding site from hluc+ver2BF7.

hluc+ver2B7 has the following sequence:

AAAGCCACCATGGAAGATGCCAAAAACATTAAGAAGGGGCCTGCTC
CCTTCTACCCTCTTGAAGATGGGACTGCTGGCGAGCAACTTCACAAA
GCTATGAAGCGGTATGCTCTTGTGCCAGGGACAATTGCGTTCACGGA
TGCTCACATTGAAGTAGACATCACATACGCTGAGTATTTTGAGATGTC

GGTGCGGCTGGCAGAAGCTATGAAGCGCTATGGGCTGAATACAAACC
ATAGAATTGTAGTGTGCAGTGAGAACTCGTTGCAGTTCTTTATGCCCG
TGCTGGGGGCTCTCTTCATCGGGGTGGCTGTGGCTCCTGCTAACGACA
TCTACAACGAGCGAGAGCTGTTGAACTCGATGGGGATCTCTCAGCCT
ACAGTGGTGTTTGTGAGTAAGAAAGGGCTTCAAAAGATTCTCAATGT
GCAAAAGAAGCTGCCTATTATACAAAAGATTATTATTATGGACTCTA
AGACAGACTACCAGGGGTTTCAGTCCATGTACACATTTGTAACCTCTC
ATCTGCCTCCTGGCTTCAACGAGTACGACTTCGTGCCCGAGTCTTTCG
ACAGGGACAAAACGATTGCTCTGATCATGAACAGCTCCGGGTCTACC
GGGCTGCCTAAGGGTGTAGCTCTGCCCCATCGAACAGCTTGTGTGAG
ATTCTCTCATGCCAGGGACCCGATCTTTGGAAACCAGATCATCCCTGA
CACTGCTATTCTGTCGGTGGTGCCCTTTCATCATGGGTTTGGGATGTT
CACAACACTGGGATACCTCATTTGCGGGTTTAGAGTGGTGCTCATGTA
TAGGTTTGAAGAAGAACTATTCCTACGCTCTTTGCAAGATTATAAGAT
TCAGTCTGCTCTGCTGGTGCCAACACTATTCTCTTTTTTTGCTAAGTCT
ACGCTCATAGACAAGTATGACTTGTCCAACTTGCACGAGATTGCTTCT
GGCGGAGCACCTCTGTCTAAGGAGGTAGGTGAGGCTGTGGCTAAGCG
CTTTCATCTGCCTGGTATCAGACAGGGGTACGGGCTAACAGAAACAA
CTTCTGCTATTCTGATTACACCAGAGGGCGATGACAAACCCGGGGCT
GTAGGGAAAGTGGTGCCCTTTTTTGAAGCCAAAGTAGTTGATCTTGAT
ACCGGTAAGACACTAGGGGTGAACCAGCGTGGTGAACTGTGTGTGCG
GGGCCCTATGATTATGTCGGGGTACGTTAACAACCCCGAAGCTACAA
ATGCTCTCATAGACAAGGACGGGTGGCTTCATAGCGGCGACATTGCC
TACTGGGACGAGGATGAGCATTTCTTCATCGTGGACAGACTGAAGTC
GTTGATCAAATACAAGGGGTATCAAGTAGCTCCTGCCGAGCTTGAGT
CCATTCTGCTTCAACACCCCAATATCTTCGATGCTGGGGTGGCTGGGC
TGCCTGATGATGATGCTGGAGAGCTGCCTGCTGCTGTAGTAGTGCTTG
AGCATGGTAAGACAATGACAGAGAAGGAGATCGTGGATTATGTGGCT
TCACAAGTGACAACAGCTAAGAAACTCCGAGGTGGCGTTGTGTTTGT
GGATGAGGTGCCTAAAGGGCTCACTGGCAAGCTGGATGCCAGAAAA
ATTCGAGAGATTCTCATTAAGGCTAAGAAGGGTGGAAAGATTGCTGT
GTAATAGTTCTAGA (SEQ ID NO:94)

hluc+ver2B8 has the following sequence

AAAGCCACCATGGAaGATGCcAAaAAcATTAAGAAGGGGCCTGCTCCc
TTcTAcCCTCTtGAaGATGGGACtGCtGGcGAGCAaCTtCAcAAaGCTATGA
AGcGgTATGCTCTtGTGCCaGGgACAATTGCgTTcACgGATGCTCAcATTG
AaGTaGAcATcACATAcGCTGAGTATTTTGAGATGTCgGTGcGgCTGGCa
GAaGCTATGAAGcGcTATGGGCTGAATACAAAcCATAGAATTGTaGTGT
GcagTGAGAAcTCgtTGCAGTTcTTTATGCCcGTGCTGGGGGCTCTcTTcAT
cGGGGTGGCTGTGGCTCCTGCTAAcGAcATcTAcAAcGAGcGAGAGCTgt
TGAAcTCgATGGGGATcTCTCAGCCTACAGTGGTGTTTGTGagTAAGAA
aGGGCTtCAaAAGATTCTcAATGTGCAaAAGAAGCTaCCgATcATaCAaAA
GATcATcATcATGGAtagcAAGACcGAcTAcCAGGGGTTTCAGTCcATGTA
cACATTTGTaACcTCTCATCTGCCTCCTGGcTTcAAcGAGTAcGAcTTcGT
GCCcGAGTCTTTcGAcAGgGAcAAaACgATTGCTCTGATcATGAAcagcTCc
GGGTCTACcGGGCTGCCTAAGGGtGTaGCTCTGCCcCATcGAACAGCTT
GTGTGAGATTcTCTCATGCcAGgGAcCCgATcTTtGGaAAcCAGATcATcC
CTGAcACtGCTATTCTGTCgGTgGTGCCcTTTCATCATGGGTTTGGGATG
TTcACAACACTGGGaTAccTcATtTGcGGGTTTAGAGTGGTGCTcATGTAT
AGgTTTGAaGAaGAaCTaTTccTacGcTCTtTGCAaGATTATAAGATTCAGT
CTGCTCTGCTGGTGCCaACACTaTTcTCTTTTTTTGCTAAGTCTACgCTcA
TaGAcAAGTATGActTGTCcAActTGCAcGAGATTGCTTCTGGcGGaGCaCC
TCTGTCTAAGGAGGTaGGtGAGGCTGTGGCTAAGcGcTTTCATCTGCCT
GGtATcAGACAGGGGTAcGGGCTaACAGAaACAACtTCTGCTATTCTGAT
TACACCaGAGGGcGATGAcAAaCCtGGGGCTGTaGGGAAaGTGGTGCCcT
TTTTTGAaGCcAAaGTaGTtGATCTtGATACcGGtAAGACACTaGGGGTGA
AcCAGcGtGGtGAaCTGTGTGTGcGgGGcCCTATGATTATGTCgGGGTAcG
TtAAcAAcCCcGAaGCTACAAATGCTCTcATaGAcAAGGAcGGgTGGcTtC
ATagcGGcGAcATTGCcTAcTGGGAcGAGGATGAGCATTTcTTcATcGTGG
AcAGACTGAAGTCgtTGATcAAaTAcAAGGGGTATCAaGTaGCTCCTGCc
GAGCTtGAgTCcATTCTGCTtCAaCAcCCcAAtATcTTcGATGCTGGGGTGG
CTGGGCTGCCTGATGATGATGCTGGaGAGcTGCCTGCTGCTGTaGTaGT
GCTtGAGCAtGGtAAGACAATGACAGAGAAGGAGATcGTGGATTATGT
GGCTTCaCAaGTGACAACAGCTAAGAAaCTccGAGGtGGcGTtGTGTTTG
TGGATGAGGTGCCTAAaGGGCTcACtGGcAAGCTGGATGCcAGAAAaAT

TcGAGAGATTCTcATTAAGGCTAAGAAGGGtGGaAAGATTGCTGTGTA
ATAgTTCTAGA (SEQ ID NO:31).

hluc+ver2BF8 was modified to yield hluc+ver2BF9.

hluc+ver2B9 has the following sequence

AAAGCCACCATGGAaGATGCcAAaAAcATTAAGAAGGGGCCTGCTCCc
TTcTAcCCTCTtGAaGATGGGACtGCtGGcGAGCAaCTtCAcAAaGCTATGA
AGcGgTATGCTCTtGTGCCaGGgACAATTGCgTTcACgGATGCTCAcATTG
AaGTaGAcATcACATAcGCTGAGTATTTTGAGATGTCgGTGcGgCTGGCa
GAaGCTATGAAGcGcTATGGGCTGAATACAAAcCATAGAATTGTaGTGT
GcagTGAGAAcTCgtTGCAGTTcTTTATGCCcGTGCTGGGGGCTCTcTTcAT
tGGGGTGGCTGTGGCTCCTGCTAAtGAcATcTAcAAcGAGcGAGAGCTgtT
GAAcagtATGGGGATcTCTCAGCCTACAGTGGTGTTTGTGagTAAGAAaG
GGCTtCAaAAGATTCTcAATGTGCAaAAGAAGCTaCCgATcATaCAaAAG
ATcATcATcATGGAtagcAAGACcGAcTAcCAGGGGTTTCAGTCcATGTAc
ACATTTGTaACcTCTCATCTGCCTCCTGGcTTcAAtGAGTAtGAcTTcGTG
CCcGAGTCTTTcGAcAGgGAcAAaACgATTGCTCTGATcATGAAcagcagtG
GGTCTACcGGGCTGCCTAAGGGtGTaGCTCTGCCcCATcGAACAGCTTG
TGTGAGATTcTCTCATGCcAGgGAcCCgATcTTtGGaAAcCAGATcATcCCT
GAcACtGCTATTCTGTCgGTgGTGCCcTTTCATCATGGGTTTGGGATGTT
cACAACACTGGGaTAccTcATtTGcGGGTTTAGAGTGGTGCTcATGTATA
GgTTTGAaGAaGAaCTaTTccTacGcTCTtTGCAaGATTATAAGATTCAGTC
TGCTCTGCTGGTGCCaACACTaTTcTCTTTTTTTGCTAAGTCTACgCTcAT
aGAcAAGTATGActTGTCcAActTGCAcGAGATTGCTTCTGGcGGaGCaCCT
CTGTCTAAGGAGGTaGGtGAGGCTGTGGCTAAGcGcTTTCATCTGCCTG
GtATcAGACAGGGGTAcGGGCTaACAGAaACAACtTCTGCTATTCTGATT
ACACCaGAGGGcGATGAcAAaCCtGGGGCTGTaGGGAAaGTGGTGCCcTT
TTTTGAaGCcAAaGTaGTtGATCTtGATACcGGtAAGACACTaGGGGTGAA
cCAGaGaGGtGAatTGTGTGTGaGgGGcCCTATGATTATGTCgGGGTAcGTt
AAcAAcCCcGAaGCTACAAATGCTCTcATaGAcAAGGAcGGgTGGcTtCAT
agtGGaGAtATTGCcTAcTGGGAtGAaGATGAGCATTTcTTcATcGTGGAcA
GACTGAAGTCgtTGATcAAaTAcAAGGGGTATCAaGTaGCTCCTGCcGAG

CTtGAgTCcATTCTGCTtCAaCAcCCcAAtATcTTcGATGCTGGGGTGGCTG
GGCTGCCTGATGATGATGCTGGaGAGcTGCCTGCTGCTGTaGTaGTGCTt
GAGCAtGGtAAGACAATGACAGAGAAGGAGATcGTGGATTATGTGGCT
TCaCAaGTGACAACAGCTAAGAAaCTccGAGGtGGcGTtGTGTTTGTGGA
TGAGGTGCCTAAaGGGCTcACtGGcAAGCTGGATGCcAGAAAaATTcGA
GAGATTCTcATTAAGGCTAAGAAGGGtGGaAAGATTGCTGTGTAATAgT
TCTAGA (SEQ ID NO:32).

The *BglI* sequence in hluc+ver2BF9 was removed resulting in hluc+ver2BF10. hluc+ver2BF10 demonstrated poor expression.

hluc+ver2B10 has the following sequence
AAAGCCACCATGGAaGATGCcAAaAAcATTAAGAAGGGGCCTGCTCCc
TTcTAcCCTCTtGAaGATGGGACtGCtGGcGAGCAaCTtCAcAAaGCTATGA
AGcGgTATGCTCTtGTGCCaGGgACAATTGCgTTcACgGATGCTCAcATTG
AaGTaGAcATcACATAcGCTGAGTATTTTGAGATGTCgGTGcGgCTGGCa
GAaGCTATGAAGcGcTATGGGCTGAATACAAAcCATAGAATTGTaGTGT
GcagTGAGAAcTCgtTGCAGTTcTTTATGCCcGTGCTGGGGGCTCTcTTcAT
tGGGGTGGCTGTGGCTCCTGCTAAtGAcATcTAcAAcGAGcGAGAGCTgtT
GAAcagtATGGGGATcTCTCAGCCTACAGTGGTGTTTGTGagTAAGAAaG
GGCTtCAaAAGATTCTcAATGTGCAaAAGAAGCTaCCgATcATaCAaAAG
ATcATcATcATGGAtagcAAGACcGAcTAcCAGGGGTTTCAGTCcATGTAc
ACATTTGTaACcTCTCATCTGCCTCCTGGcTTcAAtGAGTAtGAcTTcGTG
CCcGAGTCTTTcGAcAGgGAcAAaACgATTGCTCTGATcATGAAcagcagtG
GGTCTACcGGGCTGCCTAAGGGtGTaGCTCTGCCcCATcGAACAGCTTG
TGTGAGATTcTCTCATGCcAGgGAcCCgATcTTtGGaAAcCAGATcATcCCT
GAcACtGCTATTCTGTCgGTgGTGCCcTTTCATCATGGGTTTGGGATGTT
cACAACACTGGGaTAccTcATtTGcGGGTTTAGAGTGGTGCTcATGTATA
GgTTTGAaGAaGAaCTaTTccTacGcTCTtTGCAaGATTATAAGATTCAGTC
TGCTCTGCTGGTGCCaACACTaTTcTCTTTTTTTGCTAAGTCTACgCTcAT
aGAcAAGTATGActTGTCcAActTGCAcGAGATTGCTTCTGGcGGaGCaCCT
CTGTCTAAGGAGGTaGGtGAGGCTGTGGCTAAGcGcTTTCATCTGCCTG
GtATcAGACAGGGGTAcGGGCTaACAGAaACAACtTCTGCTATTCTGATT

ACACCaGAGGGcGATGAcAAaCCtGGGGCTGTaGGGAAaGTGGTGCCcTT
TTTTGAaGCcAAaGTaGTtGATCTtGATACcGGtAAGACACTaGGGGTGAA
cCAGaGaGGtGAatTGTGTGTGaGgGGcCCTATGATTATGTCgGGGTAcGTt
AAcAAcCCcGAaGCTACAAATGCTCTcATaGAcAAGGAcGGgTGGcTtCAT
agtGGaGAtATTGCcTAcTGGGAtGAaGATGAGCATTTcTTcATcGTGGAcA
GACTGAAGTCgtTGATcAAaTAcAAGGGGTATCAaGTaGCTCCTGCcGAG
CTtGAgTCcATTCTGCTtCAaCAcCCcAAtATcTTcGATGCTGGGGTGGCTG
GGCTGCCTGATGATGATGCTGGaGAGcTGCCTGCTGCTGTaGTaGTGCTt
GAGCAtGGtAAGACAATGACAGAGAAGGAGATcGTGGATTATGTGGCT
TCaCAaGTGACAACAGCTAAGAAaCTccGAGGtGGcGTtGTGTTTGTGGA
TGAGGTGCCTAAaGGaCTcACtGGcAAGCTGGATGCcAGAAAaATTcGAG
AGATTCTcATTAAGGCTAAGAAGGGtGGaAAGATTGCTGTGTAATAgTT
CTAGA (SEQ ID NO:33).

Table 11

Summary of Firefly Luciferase Constructs

Firefly luciferase Gene	Number of consensus transcription factor binding sites	Number of Promoter modules*	CG dinucleotides (possible methylation sites)
Luc+	287	7	97
hluc+ver2AF8	3	0	132
hluc+ver2BF10	3	0	43

*Promoter modules are defined as a composite regulatory element, with 2 TFBS separated by a spacer, which has been shown to exhibit synergistic or antagonistic function.

Example 4

Synthetic Selectable Polypeptide Genes

Design Process

Define sequences

Protein sequence that should be maintained:

- Neo: from *neo* gene of pCI-neo (Promega) (SEQ ID NO:1)
- Hyg: from *hyg* gene of pcDNA3.1/Hygro (Invitrogen) (SEQ ID NO:6)

DNA flanking regions for starting sequence:

- 5′ end: Kozak sequence from *neo* gene of pCI-neo (GCCACCATGA; SEQ ID NO:34)), *Pfl*MI site (*CCANNNNNTGG*; SEQ ID NO:35), add Ns at end (to avoid search algorithm errors & keep ORF1):
  neo/hyg: NNNNN*CCAnnnnnTGG*GCCACC-**ATG**-G (SEQ ID NO:36)

Change: replace *Pfl*MI with *Sbf*I (CCTGCAGG)

- 3′ end: two stop codons (at least one TAA), *Pfl*MI site (not compatible with that at 5′ end to allow directional cloning), add Ns at end (to avoid search algorithm errors):
  neo/hyg: **TAATAA***CCAnnnnnTGG*NNN (SEQ ID NO:37)

Change: replace *Pfl*MI with *Afl*II (CTTAAG)

Define codon usage

Codon usage was obtained from the Codon Usage Database (http://www.kazusa.or.jp/codon/):

Based on: GenBank Release 131.0 [15 August 2002] (Nakamura et al., 2000).

Codon usage tables were downloaded for:

HS: *Homo sapiens* [gbpri] 50,031 CDS's (21,930,294 codons)

MM: *Mus musculus* [gbrod] 23,113 CDS's (10,345,401 codons)

EC: *Escherichia coli* [gbbct] 11,985 CDS's (3,688,954 codons)

EC K12: *Escherichia coli* K12 [gbbct] 4,291 CDS's (1,363,716 codons)

⇨ HS and MM were compared and found to be closely similar, use HS table

⇨ EC and EC K12 were compared and found to be closely similar, use EC K12 table

Codon selection strategy:

Overall strategy is to adapt codon usage for optimal expression in mammalian cells while avoiding low-usage *E. coli* codons. One "best" codon was selected for each amino acid and used to back-translate the desired protein sequence to yield a starting gene sequence.

Strategy A was chosen for the design of the *neo* and *hyg* genes (see Table 12). (Strategy A: Codon bias optimized: emphasis on codons showing the highest usage frequency in HS. Best codons are those with highest

usage in HS, unless a codon with slightly lower usage has substantially higher usage in *E. coli*.).

Table 12

Amino acid	Codon Choices in Examples 1-2	Codon Choices in Codon Bias Optimized Strategy A
Gly	GGC/GGT	GGC
Glu	GAG	GAG
Asp	GAC	GAC
Val	GTG/GTC	GTG
Ala	GCC/GCT	GCC
Arg	CGC/CGT	CGC
Ser	TCT/AGC	AGC
Lys	AAG	AAG
Asn	AAC	AAC
Ile	ATC/ATT	ATC
Thr	ACC/ACT	ACC
Cys	TGC	TGC
Tyr	TAC	TAC
Leu	CTG/TTG	CTG
Phe	TTC	TTC
Gln	CAG	CAG
His	CAC	CAC
Pro	CCA/CCT	CCC

Generate starting gene sequences

Use custom codon usage table in Vector NTI 8.0 (Informax) ("Strategy A")

Back-translate *neo* and *hyg* protein sequences

Neo (based on neomycin gene from Promega's pCI-neo)

MIEQDGLHAGSPAAWVERLFGYDWAQQTIGCSDAAVFRLSAQGRPVLF
VKTDLSGALNELQDEAARLSWLATTGVPCAAVLDVVTEAGRDWLLLGE
VPGQDLLSSHLAPAEKVSIMADAMRRLHTLDPATCPFDHQAKHRIERAR

TRMEAGLVDQDDLDEEHQGLAPAELFARLKARMPDGEDLVVTHGDAC
LPNIMVENGRFSGFIDCGRLGVADRYQDIALATRDIAEELGGEWADRFLV
LYGIAAPDSQRIAFYRLLDEFF (SEQ ID NO:2) and encoded by

Atgattgaacaagatggattgcacgcaggttctccggccgcttgggtggagaggctattcggctatgactgggcac
aacagacaatcggctgctctgatgccgccgtgttccggctgtcagcgcaggggcgcccggttctttttgtcaagacc
gacctgtccggtgccctgaatgaactgcaggacgaggcagcgcggctatcgtggctggccacgacgggcgttcct
tgcgcagctgtgctcgacgttgtcactgaagcgggaagggactggctgctattgggcgaagtgccggggcaggat
ctcctgtcatctcaccttgctcctgccgagaaagtatccatcatggctgatgcaatgcggcggctgcatacgcttgatc
cggctacctgcccattcgaccaccaagcgaaacatcgcatcgagcgagcacgtactcggatggaagccggtcttgt
cgatcaggatgatctggacgaagagcatcaggggctcgcgccagccgaactgttcgccaggctcaaggcgcgcat
gcccgacggcgaggatctcgtcgtgacccatggcgatgcctgcttgccgaatatcatggtggaaaatggccgctttt
ctggattcatcgactgtggccggctgggtgtggcggaccgctatcaggacatagcgttggctacccgtgatattgctg
aagagcttggcggcgaatgggctgaccgcttcctcgtgctttacggtatcgccgctcccgattcgcagcgcatcgcc
ttctatcgccttcttgacgagttcttctga (SEQ ID NO:1)

Hyg (based on hygromycin gene from Invitrogen's pcDNA3.1/Hygro)
MKKPELTATSVEKFLIEKFDSVSDLMQLSEGEESRAFSFDVGGRGYVLRV
NSCADGFYKDRYVYRHFASAALPIPEVLDIGEFSESLTYCISRRAQGVTLQ
DLPETELPAVLQPVAEAMDAIAAADLSQTSGFGPFGPQGIGQYTTWRDFI
CAIADPHVYHWQTVMDDTVSASVAQALDELMLWAEDCPEVRHLVHAD
FGSNNVLTDNGRITAVIDWSEAMFGDSQYEVANIFFWRPWLACMEQQT
RYFERRHPELAGSPRLRAYMLRIGLDQLYQSLVDGNFDDAAWAQGRCD
AIVRSGAGTVGRTQIARRSAAVWTDGCVEVLADSGNRRPSTRPRAKE
(SEQ ID NO:7) encoded by

Atgaaaaagcctgaactcaccgcgacgtctgtcgagaagtttctgatcgaaaagttcgacagcgtctccgacctgat
gcagctctcggagggcgaagaatctcgtgctttcagcttcgatgtaggagggcgtggatatgtcctgcgggtaaata
gctgcgccgatggtttctacaaagatcgttatgtttatcggcactttgcatcggccgcgctcccgattccggaagtgctt
gacattggggaattcagcgagagcctgacctattgcatctcccgccgtgcacagggtgtcacgttgcaagacctgcc
tgaaaccgaactgcccgctgttctgcagccggtcgcggaggccatggatgcgatcgctgcggccgatcttagccag
acgagcgggttcggcccattcggaccgcaaggaatcggtcaatacactacatggcgtgatttcatatgcgcgattgc
tgatccccatgtgtatcactggcaaactgtgatggacgacaccgtcagtgcgtccgtcgcgcaggctctcgatgagc
tgatgctttgggccgaggactgccccgaagtccggcacctcgtgcacgcggatttcggctccaacaatgtcctgacg

gacaatggccgcataacagcggtcattgactggagcgaggcgatgttcgggattcccaatacgaggtcgccaac
atcttcttctggaggccgtggttggcttgtatggagcagcagacgcgctacttcgagcggaggcatccggagcttgc
aggatcgccgcggctccgggcgtatatgctccgcattggtcttgaccaactctatcagagcttggttgacggcaatttc
gatgatgcagcttgggcgcagggtcgatgcgacgcaatcgtccgatccggagccgggactgtcgggcgtacacaa
atcgcccgcagaagcgcggccgtctggaccgatggctgtgtagaagtactcgccgatagtggaaaccgacgcccc
agcactcgtccgagggcaaaggaat (SEQ ID NO:6).

Table 13

Nomenclature of exemplary neo and hyg gene versions

Gene name	Description
neo	from pCI-neo (Promega)
hneo	humanized (codon usage strategy A) ORF
hneo-F	humanized ORF with 5′ and 3′ flanking regions
hneo-1F	humanized ORF with 5′ and 3′ flanking regions after first removal of undesired sequence matches
hneo-2F	humanized ORF with 5′ and 3′ flanking regions after second removal of undesired sequence matches
hneo-3F	humanized ORF with 5′ and 3′ flanking regions after third removal of undesired sequence matches
hneo-3FB	Changed 5′ and 3′ flanking cloning sites
hyg	from pcDNA3.1/Hygro (Invitrogen)
hhyg	humanized (codon usage strategy A) ORF
hhyg-F	humanized ORF with 5′ and 3′ flanking regions
hhyg-1F	humanized ORF with 5′ and 3′ flanking regions after first removal of undesired sequence matches
hhyg-2F	humanized ORF with 5′ and 3′ flanking regions after second removal of undesired sequence matches
hhyg-3F	humanized ORF with 5′ and 3′ flanking regions after third removal of undesired sequence matches
hhyg-3FB	Changed 5′ and 3′ flanking cloning sites

"h" indicates humanized codons, "F" indicates presence of 5' and 3' flanking sequences.

Create starting (codon-optimized) gene sequences:

hneo (humanized starting gene sequence without flanking regions in hneo-F)

CCACTCAGTGGCCACCATGATCGAGCAGGACGGCCTGCACGCCGGCA
GCCCCGCCGCCTGGGTGGAGCGCCTGTTCGGCTACGACTGGGCCCAG
CAGACCATCGGCTGCAGCGACGCCGCCGTGTTCCGCCTGAGCGCCCA
GGGCCGCCCCGTGCTGTTCGTGAAGACCGACCTGAGCGGCGCCCTGA
ACGAGCTGCAGGACGAGGCCGCCCGCCTGAGCTGGCTGGCCACCACC
GGCGTGCCCTGCGCCGCCGTGCTGGACGTGGTGACCGAGGCCGGCCG
CGACTGGCTGCTGCTGGGCGAGGTGCCCGGCCAGGACCTGCTGAGCA
GCCACCTGGCCCCCGCCGAGAAGGTGAGCATCATGGCCGACGCCATG
CGCCGCCTGCACACCCTGGACCCCGCCACCTGCCCCTTCGACCACCA
GGCCAAGCACCGCATCGAGCGCGCCCGCACCCGCATGGAGGCCGGC
CTGGTGGACCAGGACGACCTGGACGAGGAGCACCAGGGCCTGGCCC
CCGCCGAGCTGTTCGCCCGCCTGAAGGCCCGCATGCCCGACGGCGAG
GACCTGGTGGTGACCCACGGCGACGCCTGCCTGCCCAACATCATGGT
GGAGAACGGCCGCTTCAGCGGCTTCATCGACTGCGGCCGCCTGGGCG
TGGCCGACCGCTACCAGGACATCGCCCTGGCCACCCGCGACATCGCC
GAGGAGCTGGGCGGCGAGTGGGCCGACCGCTTCCTGGTGCTGTACGG
CATCGCCGCCCCCGACAGCCAGCGCATCGCCTTCTACCGCCTGCTGG
ACGAGTTCTTCTAATAACCAGTCTCTGG (SEQ ID NO:3).

hhyg (humanized starting gene sequence without flanking regions)

CCACTCAGTGGCCACCATGAAGAAGCCCGAGCTGACCGCCACCAGCG
TGGAGAAGTTCCTGATCGAGAAGTTCGACAGCGTGAGCGACCTGATG
CAGCTGAGCGAGGGCGAGGAGAGCCGCGCCTTCAGCTTCGACGTGG
GCGGCCGCGGCTACGTGCTGCGCGTGAACAGCTGCGCCGACGGCTTC
TACAAGGACCGCTACGTGTACCGCCACTTCGCCAGCGCCGCCCTGCC
CATCCCCGAGGTGCTGGACATCGGCGAGTTCAGCGAGAGCCTGACCT
ACTGCATCAGCCGCCGCGCCCAGGGCGTGACCCTGCAGGACCTGCCC
GAGACCGAGCTGCCCGCCGTGCTGCAGCCCGTGGCCGAGGCCATGGA

CGCCATCGCCGCCGCCGACCTGAGCCAGACCAGCGGCTTCGGCCCCT
TCGGCCCCCAGGGCATCGGCCAGTACACCACCTGGCGCGACTTCATC
TGCGCCATCGCCGACCCCCACGTGTACCACTGGCAGACCGTGATGGA
CGACACCGTGAGCGCCAGCGTGGCCCAGGCCCTGGACGAGCTGATGC
TGTGGGCCGAGGACTGCCCCGAGGTGCGCCACCTGGTGCACGCCGAC
TTCGGCAGCAACAACGTGCTGACCGACAACGGCCGCATCACCGCCGT
GATCGACTGGAGCGAGGCCATGTTCGGCGACAGCCAGTACGAGGTGG
CCAACATCTTCTTCTGGCGCCCCTGGCTGGCCTGCATGGAGCAGCAG
ACCCGCTACTTCGAGCGCCGCCACCCCGAGCTGGCCGGCAGCCCCCG
CCTGCGCGCCTACATGCTGCGCATCGGCCTGGACCAGCTGTACCAGA
GCCTGGTGGACGGCAACTTCGACGACGCCGCCTGGGCCCAGGGCCGC
TGCGACGCCATCGTGCGCAGCGGCGCCGGCACCGTGGGCCGCACCCA
GATCGCCCGCCGCAGCGCCGCCGTGTGGACCGACGGCTGCGTGGAGG
TGCTGGCCGACAGCGGCAACCGCCGCCCCAGCACCCGCCCCCGCGCC
AAGGAGTAATAACCAGCTCTTGG (SEQ ID NO:8).

Programs and databases used for identification and removal of sequence motifs

All from Genomatix Software GmbH (Munich, Germany, http://www.genomatix.de):

GEMS Launcher Release 3.5.2 (June 2003)

MatInspector professional Release 6.2.1 June 2003

Matrix Family Library Ver 3.1.2 June 2003 (incl. 318 vertebrate matrices in 128 families)

ModelInspector professional Release 4.8 October 2002

Model Library Ver 3.1 March 2003 (226 modules)

SequenceShaper tool

User Defined Matrices

Sequence motifs to remove from starting gene sequences

(In order of priority)

Restriction enzyme recognition sequences:

See user-defined matrix subset *neo* and *hyg*. Same as those used for design of hluc+ version 2.0

Generally includes those required for cloning (pGL4) or commonly used

for cloning

Change: also *Sbf*I, *Afl*II, *Acc*III

Transcription factor binding sequences:

Promoter modules (2 TF binding sites with defined orientation) with default score or greater

Vertebrate TF binding sequences with score of at least core=0.75 / matrix=optimized

Eukaryotic transcription regulatory sites:

Kozak sequence

Splice donor / acceptor sequences in (+) strand

PolyA addition sequences in (+) strand

Prokaryotic transcription regulatory sequences:

E. coli promoters

E. coli RBS (if less than 20 bp upstream of Met codon)

User-defined matrix subset "*neo+hyg*"

Format: Matrix name (core similarity threshold / matrix similarity threshold)

- U$AatII (0.75/1.00)
- U$BamHI (0.75/1.00)
- U$BglI (0.75/1.00)
- U$BglII (0.75/1.00)
- U$BsaI (0.75/1.00)
- U$BsmAI (0.75/1.00)
- U$BsmBI (0.75/1.00)
- U$BstEII (0.75/1.00)
- U$BstXI (0.75/1.00)
- U$Csp45I (0.75/1.00)
- U$CspI (0.75/1.00)

- U$EC-P-10 (1.00/Optimized)
- U$EC-P-35 (1.00/Optimized)
- U$EC-Prom (1.00/Optimized)
- U$EC-RBS (0.75/1.00)
- U$EcoRI (0.75/1.00)
- U$HindIII (0.75/1.00)
- U$Kozak (0.75/Optimized)
- U$KpnI (0.75/1.00)
- U$MluI (0.75/1.00)
- U$NcoI (0.75/1.00)
- U$NdeI (0.75/1.00)
- U$NheI (0.75/1.00)
- U$NotI (0.75/1.00)
- U$NsiI (0.75/1.00)
- U$PflMI (0.75/1.00)
- U$PmeI (0.75/1.00)
- U$PolyAsig (0.75/1.00)
- U$PstI (0.75/1.00)
- U$SacI (0.75/1.00)
- U$SacII (0.75/1.00)
- U$SalI (0.75/1.00)
- U$SfiI (0.75/1.00)
- U$SgfI (0.75/1.00)

- U$SmaI (0.75/1.00)
- U$SnaBI (0.75/1.00)
- U$SpeI (0.75/1.00)
- U$Splice-A (0.75/Optimized)
- U$Splice-D (0.75/Optimized)
- U$XbaI (0.75/1.00)
- U$XcmI (0.75/1.00)
- U$XhoI (0.75/1.00)
- ALL vertebrates.lib (0.75/Optimized)

User-defined matrix subset "*neo+hyg*-EC"

Format: Matrix name (core similarity threshold / matrix similarity threshold)

- U$AatII (0.75/1.00)
- U$BamHI (0.75/1.00)
- U$BglI (0.75/1.00)
- U$BglII (0.75/1.00)
- U$BsaI (0.75/1.00)
- U$BsmAI (0.75/1.00)
- U$BsmBI (0.75/1.00)
- U$BstEII (0.75/1.00)
- U$BstXI (0.75/1.00)
- U$Csp45I (0.75/1.00)
- U$CspI (0.75/1.00)
- U$EcoRI (0.75/1.00)

- U$HindIII (0.75/1.00)
- U$Kozak (0.75/Optimized)
- U$KpnI (0.75/1.00)
- U$MluI (0.75/1.00)
- U$NcoI (0.75/1.00)
- U$NdeI (0.75/1.00)
- U$NheI (0.75/1.00)
- U$NotI (0.75/1.00)
- U$NsiI (0.75/1.00)
- U$PflMI (0.75/1.00)
- U$PmeI (0.75/1.00)
- U$PolyAsig (0.75/1.00)
- U$PstI (0.75/1.00)
- U$SacI (0.75/1.00)
- U$SacII (0.75/1.00)
- U$SalI (0.75/1.00)
- U$SfiI (0.75/1.00)
- U$SgfI (0.75/1.00)
- U$SmaI (0.75/1.00)
- U$SnaBI (0.75/1.00)
- U$SpeI (0.75/1.00)
- U$Splice-A (0.75/Optimized)
- U$Splice-D (0.75/Optimized)

- U$XbaI (0.75/1.00)
- U$XcmI (0.75/1.00)
- U$XhoI (0.75/1.00)
- ALL vertebrates.lib (0.75/Optimized)

User-defined matrix subset "pGL4-072503"

Format: Matrix name (core similarity threshold / matrix similarity threshold)

- U$AatII (0.75/1.00)
- U$AccIII (0.75/1.00)
- U$AflII (0.75/1.00)
- U$BamHI (0.75/1.00)
- U$BglI (0.75/1.00)
- U$BglII (0.75/1.00)
- U$BsaI (0.75/1.00)
- U$BsmAI (0.75/1.00)
- U$BsmBI (0.75/1.00)
- U$BstEII (0.75/1.00)
- U$BstXI (0.75/1.00)
- U$Csp45I (0.75/1.00)
- U$CspI (0.75/1.00)
- U$EC-P-10 (1.00/Optimized)
- U$EC-P-35 (1.00/Optimized)
- U$EC-Prom (1.00/Optimized)
- U$EC-RBS (0.75/1.00)

- U$EcoRI (0.75/1.00)
- U$HindIII (0.75/1.00)
- U$Kozak (0.75/Optimized)
- U$KpnI (0.75/1.00)
- U$MluI (0.75/1.00)
- U$NcoI (0.75/1.00)
- U$NdeI (0.75/1.00)
- U$NheI (0.75/1.00)
- U$NotI (0.75/1.00)
- U$NsiI (0.75/1.00)
- U$PflMI (0.75/1.00)
- U$PmeI (0.75/1.00)
- U$PolyAsig (0.75/1.00)
- U$PstI (0.75/1.00)
- U$SacI (0.75/1.00)
- U$SacII (0.75/1.00)
- U$SalI (0.75/1.00)
- U$SbfI (0.75/1.00)
- U$SfiI (0.75/1.00)
- U$SgfI (0.75/1.00)
- U$SmaI (0.75/1.00)
- U$SnaBI (0.75/1.00)
- U$SpeI (0.75/1.00)

- U$Splice-A (0.75/Optimized)
- U$Splice-D (0.75/Optimized)
- U$XbaI (0.75/1.00)
- U$XcmI (0.75/1.00)
- U$XhoI (0.75/1.00)
- ALL vertebrates.lib

Strategy for removal of sequence motifs

The undesired sequence motifs specified above were removed from the starting gene sequence by selecting alternate codons that allowed retention of the specified protein and flanking sequences. Alternate codons were selected in a way to conform to the overall codon selection strategy as much as possible.

General steps:

- Identify undesired sequence matches with MatInspector using matrix family subset "neo+hyg" or "neo+hyg-EC" and with ModelInspector using default settings.
- Identify possible replacement codons to remove undesired sequence matches with SequenceShaper (keep ORF).
- Incorporate changes into a new version of the synthetic gene sequence and re-analyze with MatInspector and ModelInspector.

Specific steps:

- First try to remove undesired sequence matches using subset "neo+hyg-EC" and SequenceShaper default remaining thresholds (0.70/Opt-0.20).
- For sequence matches that cannot be removed with this approach use lower SequenceShaper remaining thresholds (e.g. 0.70/Opt-0.05).
- For sequence matches that still cannot be removed, try different combinations of manually chosen replacement codons (especially if more than 3 base changes might be needed). If that introduces new sequence

matches, try to remove those using the steps above (a different starting sequence sometimes allows a different removal solution).

- Use subset "neo+hyg" to check whether problematic *E. coli* sequence matches were introduced, and if so try to remove them using an analogous approach to that described above for non *E. coli* sequences.

Use an analogous strategy for the flanking (non-ORF) sequences.

Final check with subset "pGL4-072503" after change in flanking cloning sites

After codon optimizing *neo* and *hyg*, hneo and hhyg were obtained. Regulatory sequences were removed from hneo and hhyg yielding hneo-1F and hhyg-1F (the corresponding sequences without flanking regions are SEQ ID Nos. 38 and 30, respectively). Regulatory sequences were removed from hneo-1F and hhyg-1F yielding hneo-2F and hhyg-2F (the corresponding sequences without flanking regions are SEQ ID Nos. 39 and 42, respectively). Regulatory sequences were removed from hneo-2F and hhyg-2F yielding hneo-3F and hhyg-3F. Hneo-3F and hhyg-3F were further modified by altering 5′ and 3′ cloning sites yielding hneo-3FB and hhyg-3FB:

hneo-3 (after 3rd round of sequence removal, subset neo+hyg) has the following sequence:

CCACTCcGTGGCCACCATGATCGAaCAaGACGGCCTcCAtGCtGGCAGtC
CCGCaGCtTGGGTcGAaCGCtTGTTCGGgTACGACTGGGCCCAGCAGAC
CATCGGaTGtAGCGAtGCgGCCGTGTTCCGtCTaAGCGCtCAaGGCCGgCC
CGTGCTGTTCGTGAAGACCGACCTGAGCGGCGCCCTGAACGAGCTtCA
aGACGAGGCtGCCCGCCTGAGCTGGCTGGCCACCACCGGtGTaCCCTGC
GCCGCtGTGtTGGAtGTtGTGACCGAaGCCGGCCGgGACTGGCTGCTGCT
GGGCGAGGTcCCtGGCCAGGAtCTGCTGAGCAGCCACCTtGCCCCCGCt
GAGAAGGTttcCATCATGGCCGAtGCaATGCGgCGCCTGCACACCCTGG
ACCCCGCtACaTGCCCCTTCGACCACCAGGCtAAGCAtCGgATCGAGCGt
GCtCGgACCCGCATGGAGGCCGGCCTGGTGGACCAGGACGACCTGGA
CGAGGAGCAtCAGGGCCTGGCCCCCGCtGAaCTGTTCGCCCGCCTGAAa
GCCCGCATGCCgGACGGtGAGGACCTGGTtGTGACaCAtGGtGAtGCCTG
CCTcCCtAACATCATGGTcGAGAAtGGcCGCTTCtcCGGCTTCATCGACTG

CGGtCGCCTaGGaGTtGCCGACCGCTACCAGGACATCGCCCTGGCCACC
CGCGACATCGCtGAGGAGCTtGGCGGCGAGTGGGCCGACCGCTTCtTaG
TctTGTACGGCATCGCaGCtCCCGACAGCCAGCGCATCGCCTTCTACCG
CCTGCTcGACGAGTTCTTtTAATGACCAGgCTCTGG (SEQ ID NO:4);

hneo-3FB (change *Pfl*MI sites to *Sbf*I at 5' end and *Afl*II at 3' end) has the following sequence:

cctgcaggCCACCATGATCGAACAAGACGGCCTCCATGCTGGCAGTCCCG
CAGCTTGGGTCGAACGCTTGTTCGGGTACGACTGGGCCCAGCAGACC
ATCGGATGTAGCGATGCGGCCGTGTTCCGTCTAAGCGCTCAAGGCCG
GCCCGTGCTGTTCGTGAAGACCGACCTGAGCGGCGCCCTGAACGAGC
TTCAAGACGAGGCTGCCCGCCTGAGCTGGCTGGCCACCACCGGTGTA
CCCTGCGCCGCTGTGTTGGATGTTGTGACCGAAGCCGGCCGGGACTG
GCTGCTGCTGGGCGAGGTCCCTGGCCAGGATCTGCTGAGCAGCCACC
TTGCCCCCGCTGAGAAGGTTTCCATCATGGCCGATGCAATGCGGCGC
CTGCACACCCTGGACCCCGCTACATGCCCCTTCGACCACCAGGCTAA
GCATCGGATCGAGCGTGCTCGGACCCGCATGGAGGCCGGCCTGGTGG
ACCAGGACGACCTGGACGAGGAGCATCAGGGCCTGGCCCCCGCTGA
ACTGTTCGCCCGCCTGAAAGCCCGCATGCCGGACGGTGAGGACCTGG
TTGTGACACATGGTGATGCCTGCCTCCCTAACATCATGGTCGAGAAT
GGCCGCTTCTCCGGCTTCATCGACTGCGGTCGCCTAGGAGTTGCCGAC
CGCTACCAGGACATCGCCCTGGCCACCCGCGACATCGCTGAGGAGCT
TGGCGGCGAGTGGGCCGACCGCTTCTTAGTCTTGTACGGCATCGCAG
CTCCCGACAGCCAGCGCATCGCCTTCTACCGCCTGCTCGACGAGTTCT
TTTAATGAgcttaag (SEQ ID NO:5);

hhyg-3 (after 3rd round of sequence removal, subset neo+hyg) has the following sequence:

CCACTCcGTGGCCACCATGAAGAAGCCCGAGCTGACCGCtACCAGCGT
tGAaAAaTTtCTcATCGAGAAGTTCGACAGtGTGAGCGACCTGATGCAGt
TgtcgGAGGGCGAaGAgAGCCGaGCCTTCAGCTTCGAtGTcGGCGGaCGC
GGCTAtGTaCTGCGgGTGAAtAGCTGCGCtGAtGGCTTCTACAAaGACCG
CTACGTGTACCGCCACTTCGCCAGCGCtGCaCTaCCCATCCCCGAaGTGt
TGGACATCGGCGAGTTCAGCGAGAGCCTGACaTACTGCATCAGtaGaCG

CGCCCAaGGCGTtACtCTcCAaGACCTcCCCGAaACaGAGCTGCCtGCtGT
GtTaCAGCCtGTcGCCGAaGCtATGGAtGCtATtGCCGCCGCCGACCTcAGt
CAaACCAGCGGCTTCGGCCCaTTCGGgCCCCAaGGCATCGGCCAGTAC
ACaACCTGGCGgGAtTTCATtTGCGCCATtGCtGAtCCCCAtGTcTACCACT
GGCAGACCGTGATGGACGACACCGTGtcCGCCAGCGTaGCtCAaGCCCT
GGACGAaCTGATGCTGTGGGCCGAaGACTGtCCCGAGGTGCGCCAcCTc
GTcCAtGCCGACTTCGGCAGCAACAACGTcCTGACCGACAACGGCCGC
ATCACCGCCGTaATCGACTGGtcCGAaGCtATGTTCGGgGACAGtCAGTA
CGAGGTGGCCAACATCTTCTTCTGGCGgCCCTGGCTGGCtTGCATGGA
GCAGCAGACtCGCTACTTCGAGCGCCGgCAtCCCGAGCTGGCCGGCAG
CCCtCGtCTGCGaGCCTACATGCTGCGCATCGGCCTGGAtCAGCTcTACC
AGAGCCTcGTGGACGGCAACTTCGACGAtGCtGCCTGGGCtCAaGGCCG
CTGCGAtGCCATCGTcCGCAGCGGgGCCGGCACCGTcGGtCGCACaCAaA
TCGCtCGCCGgAGCGCCGCCGTaTGGACCGACGGCTGCGTcGAGGTGCT
GGCCGACAGCGGCAACCGCCGgCCCAGtACaCGaCCgCGCGCtAAGGAG
TAgTAACCAGgctcTGG (SEQ ID NO:9); and

hhyg-3FB (change *Pfl*MI sites to *Sbf*I at 5' end and *Afl*II at 3' end) has the following sequence:

cctgcaggCCACCATGAAGAAGCCCGAGCTGACCGCTACCAGCGTTGAAA
AATTTCTCATCGAGAAGTTCGACAGTGTGAGCGACCTGATGCAGTTG
TCGGAGGGCGAAGAGAGCCGAGCCTTCAGCTTCGATGTCGGCGGACG
CGGCTATGTACTGCGGGTGAATAGCTGCGCTGATGGCTTCTACAAAG
ACCGCTACGTGTACCGCCACTTCGCCAGCGCTGCACTACCCATCCCC
GAAGTGTTGGACATCGGCGAGTTCAGCGAGAGCCTGACATACTGCAT
CAGTAGACGCGCCCAAGGCGTTACTCTCCAAGACCTCCCCGAAACAG
AGCTGCCTGCTGTGTTACAGCCTGTCGCCGAAGCTATGGATGCTATTG
CCGCCGCCGACCTCAGTCAAACCAGCGGCTTCGGCCCATTCGGGCCC
CAAGGCATCGGCCAGTACACAACCTGGCGGGATTTCATTTGCGCCAT
TGCTGATCCCCATGTCTACCACTGGCAGACCGTGATGGACGACACCG
TGTCCGCCAGCGTAGCTCAAGCCCTGGACGAACTGATGCTGTGGGCC
GAAGACTGTCCCGAGGTGCGCCACCTCGTCCATGCCGACTTCGGCAG
CAACAACGTCCTGACCGACAACGGCCGCATCACCGCCGTAATCGACT

GGTCCGAAGCTATGTTCGGGGACCAGTCAGTACGAGGTGGCCAACATC
TTCTTCTGGCGGCCCTGGCTGGCTTGCATGGAGCAGCAGACTCGCTAC
TTCGAGCGCCGGCATCCCGAGCTGGCCGGCAGCCCTCGTCTGCGAGC
CTACATGCTGCGCATCGGCCTGGATCAGCTCTACCAGAGCCTCGTGG
ACGGCAACTTCGACGATGCTGCCTGGGCTCAAGGCCGCTGCGATGCC
ATCGTCCGCAGCGGGGCCGGCACCGTCGGTCGCACACAAATCGCTCG
CCGGAGCGCCGCCGTATGGACCGACGGCTGCGTCGAGGTGCTGGCCG
ACAGCGGCAACCGCCGGCCCAGTACACGACCGCGCGCTAAGGAGTA
GTAActtaag (SEQ ID NO:10).

Analysis of hneo-3FB and hhyg-3FB

hneo-3FB had no transcription factor binding sequence, including promoter module, matches (GEMS release 3.5.2 June 2003; vertebrate TF binding sequence families (core similarity: 0.75 / matrix similarity: opt); and promoter modules (default parameters: optimized threshold or 80% of maximum score)), while hhyg-3FB had 4 transcription factor binding sequence matches remaining but no promoter modules (Table 10). The following transcription factor binding sequences were found in hhyg-3FB:

1) V$MINI

Family: Muscle Initiators (2 members)

Best match: Muscle Initiator Sequence 1

Ref: Laura L. Lopez & James W. Fickett "Muscle-Specific Regulation of Transcription: A Catalog of Regulatory Elements"

http://www.cbil.upenn.edu/MTIR/HomePage.html

Position in ORF: -7 to 11

2) V$PAX5

Family: PAX-5/PAX-9 B-cell-specific activating proteins (4 members)

Best match: B-cell-specific activating protein

Ref: MEDLINE 94010299

Position in ORF: 271 to 299

3) V$AREB

Family: Atp1a1 regulatory element binding (4 members)

Best match: AREB6

Ref: MEDLINE 96061934

Position in ORF: 310 to 322

4) V$VMYB

Family: AMV-viral myb oncogene (2 members)

Best match: v-Myb

Ref: MEDLINE 94147510

Position in ORF: 619 to 629

Other sequences remaining in hneo-3F included one *E. coli* RBS 8 bases upstream of Met (ORF position 334 to 337); hneo-3FB included a splice acceptor site (+) and *Pst*I site as part of a 5′ cloning site for *Sbf*I, and one *E. coli* RBS 8 bases upstream of Met (ORF position 334 to 337); hhyg-3F had no other sequence matches; and hhyg-3FB included a splice acceptor site (+) and *Pst*I site as part of a 5′ cloning site for *Sbf*I.

Subsequently, regulatory sequences were removed from hneo-3F and hhyg-3F yielding hneo-4 and hhyg-4. Then regulatory sequences were removed from hneo-4 yielding hneo-5.

Table 14

Gene name	TF binding sequences 5′ F / ORF / 3′ F	Promoter modules 5′ F / ORF / 3′ F
Neo	-- / 53 / --	-- / 0 / --
hneo-F	1 / 61 / 2	0 / 2 / 0
hneo-3F	0 / 0 / 0	0 / 0 / 0
hneo-3FB	0 / 0 / 0	0 / 0 / 0
Hyg	-- / 74 / --	-- / 3 / --
hhyg-F	1 / 94 / 1	0 / 4 / 0
hhyg-3F	1 / 3 / 0	0 / 0 / 0
hhyg-3FB	1 / 3 / 0	0 / 0 / 0

*Promoter modules are defined as a composite regulatory element, with 2 transcription factor binding sites separated by a spacer, which has been shown to exhibit synergistic or antagonistic function.

Table 15 summarizes the identity of various genes.

Table 15

Pairwise identity of different gene versions

Comparisons were of open reading frames (ORFs).

	neo	hneo	hneo-3	hneo-4	hneo-5	Final hNeo
Neo	--	79	78	78	78	77
hneo		--	90	90	90	89
hneo-3			--	100	99	98
hneo-4				--	99	98
hneo-5					--	99
Final hNeo						--

	hyg	hhyg	hhyg-3	hHygro	hhyg-4	Final hHyg
Hyg	--	79	78	73	76	78
hhyg		--	88	83	86	88
hhyg-3			--	94	96	98
hHygro				--	96	94
hhyg-4					--	97
Final hHyg						--

Percent Identity				
		1	2	
Divergence	1		82.2	1
	2	19.6		2
		1	2	

Synthetic puro-SEQ ID NO:11

Starting puro-SEQ ID NO:15

An expression cassette (hNeo-cassette) with a synthetic neomycin gene flanked by a SV40 promoter and a synthetic poly(A) site is shown below.

GGATCCGTTTGCGTATTGGGCGCTCTTCCGCTGATCTGCGCAGCACCA

TGGCCTGAAATAACCTCTGAAAGAGGAACTTGGTTAGCTACCTTCTG

AGGCGGAAAGAACCAGCTGTGGAATGTGTGTCAGTTAGGGTGTGGAA
AGTCCCCAGGCTCCCCAGCAGGCAGAAGTATGCAAAGCATGCATCTC
AATTAGTCAGCAACCAGGTGTGGAAAGTCCCCAGGCTCCCCAGCAGG
CAGAAGTATGCAAAGCATGCATCTCAATTAGTCAGCAACCATAGTCC
CGCCCCTAACTCCGCCCATCCCGCCCCTAACTCCGCCCAGTTCCGCCC
ATTCTCCGCCCCATGGCTGACTAATTTTTTTTATTTATGCAGAGGCCG
AGGCCGCCTCTGCCTCTGAGCTATTCCAGAAGTAGTGAGGAGGCTTT
TTTGGAGGCCTAGGCTTTTGCAAAAAGCTCGATTCTTCTGACACTAGC
GCCACCATGATCGAACAAGACGGCCTCCATGCTGGCAGTCCCGCAGC
TTGGGTCGAACGCTTGTTCGGGTACGACTGGGCCCAGCAGACCATCG
GATGTAGCGATGCGGCCGTGTTCCGTCTAAGCGCTCAAGGCCGGCCC
GTGCTGTTCGTGAAGACCGACCTGAGCGGCGCCCTGAACGAGCTTCA
AGACGAGGCTGCCCGCCTGAGCTGGCTGGCCACCACCGGCGTACCCT
GCGCCGCTGTGTTGGATGTTGTGACCGAAGCCGGCCGGGACTGGCTG
CTGCTGGGCGAGGTCCCTGGCCAGGATCTGCTGAGCAGCCACCTTGC
CCCCGCTGAGAAGGTTTCTATCATGGCCGATGCAATGCGGCGCCTGC
ACACCCTGGACCCCGCTACCTGCCCCTTCGACCACCAGGCTAAGCAT
CGGATCGAGCGTGCTCGGACCCGCATGGAGGCCGGCCTGGTGGACCA
GGACGACCTGGACGAGGAGCATCAGGGCCTGGCCCCCGCTGAACTGT
TCGCCCGACTGAAAGCCCGCATGCCGGACGGTGAGGACCTGGTTGTC
ACACACGGAGATGCCTGCCTCCCTAACATCATGGTCGAGAATGGCCG
CTTCTCCGGCTTCATCGACTGCGGTCGCCTAGGAGTTGCCGACCGCTA
CCAGGACATCGCCCTGGCCACCCGCGACATCGCTGAGGAGCTTGGCG
GCGAGTGGGCCGACCGCTTCTTAGTCTTGTACGGCATCGCAGCTCCC
GACAGCCAGCGCATCGCCTTCTACCGCTTGCTCGACGAGTTCTTTTAA
TGATCTAGAACCGGTCATGGCCGCAATAAAATATCTTTATTTTCATTA
CATCTGTGTGTTGGTTTTTTGTGTGTTCGAACTAGATGCTGTCGAC
(SEQ ID NO:44).

An expression cassette (hPuro-cassette) with a synthetic puromycin gene flanked by a SV40 promoter and a synthetic poly(A) site is shown below.

GGATCCGTTTGCGTATTGGGCGCTCTTCCGCTGATCTGCGCAGCACCA
TGGCCTGAAATAACCTCTGAAAGAGGAACTTGGTTAGCTACCTTCTG

AGGCGGAAAGAACCAGCTGTGGAATGTGTGTCAGTTAGGGTGTGGAA
AGTCCCCAGGCTCCCCAGCAGGCAGAAGTATGCAAAGCATGCATCTC
AATTAGTCAGCAACCAGGTGTGGAAAGTCCCCAGGCTCCCCAGCAGG
CAGAAGTATGCAAAGCATGCATCTCAATTAGTCAGCAACCATAGTCC
CGCCCCTAACTCCGCCCATCCCGCCCCTAACTCCGCCCAGTTCCGCCC
ATTCTCCGCCCCATGGCTGACTAATTTTTTTTATTTATGCAGAGGCCG
AGGCCGCCTCTGCCTCTGAGCTATTCCAGAAGTAGTGAGGAGGCTTT
TTTGGAGGCCTAGGCTTTTGCAAAAAGCTCGATTCTTCTGACACTAGC
GCCACCATGACCGAGTACAAGCCTACCGTGCGCCTGGCCACTCGCGA
TGATGTGCCCCGCGCCGTCCGCACTCTGGCCGCCGCTTTCGCCGACTA
CCCCGCTACCCGGCACACCGTGGACCCCGACCGGCACATCGAGCGTG
TGACAGAGTTGCAGGAGCTGTTCCTGACCCGCGTCGGGCTGGACATC
GGCAAGGTGTGGGTAGCCGACGACGGCGCGGCCGTGGCCGTGTGGA
CTACCCCCGAGAGCGTTGAGGCCGGCGCCGTGTTCGCCGAGATCGGC
CCCCGAATGGCCGAGCTGAGCGGCAGCCGCCTGGCCGCCCAGCAGCA
AATGGAGGGCCTGCTTGCCCCCCATCGTCCCAAGGAGCCTGCCTGGT
TTCTGGCCACTGTAGGAGTGAGCCCCGACCACCAGGGCAAGGGCTTG
GGCAGCGCCGTCGTGTTGCCCGGCGTAGAGGCCGCCGAACGCGCCGG
TGTGCCCGCCTTTCTCGAAACAAGCGCACCAAGAAACCTTCCATTCTA
CGAGCGCCTGGGCTTCACCGTGACCGCCGATGTCGAGGTGCCCGAGG
GACCTAGGACCTGGTGTATGACACGAAAACCTGGCGCCTAATGATCT
AGAACCGGTCATGGCCGCAATAAAATATCTTTATTTTCATTACATCTG
TGTGTTGGTTTTTTGTGTGTTCGAACTAGATGCTGTCGAC (SEQ ID
NO:11);

hpuro:
GCTAGCGCCACCATGACCGAGTACAAGCCCACCGTGCGCCTGGCCAC
CCGCGACGACGTGCCCCGCGCCGTGCGCACCCTGGCCGCCGCCTTCG
CCGACTACCCCGCCACCCGCCACACCGTGGACCCCGACCGCCACATC
GAGCGCGTGACCGAGCTGCAGGAGCTGTTCCTGACCCGCGTGGGCCT
GGACATCGGCAAGGTGTGGGTGGCCGACGACGGCGCCGCCGTGGCC
GTGTGGACCACCCCCGAGAGCGTGGAGGCCGGCGCCGTGTTCGCCGA
GATCGGCCCCCGCATGGCCGAGCTGAGCGGCAGCCGCCTGGCCGCCC

AGCAGCAGATGGAGGGCCTGCTGGCCCCCCACCGCCCCAAGGAGCCC
GCCTGGTTCCTGGCCACCGTGGGCGTGAGCCCCGACCACCAGGGCAA
GGGCCTGGGCAGCGCCGTGGTGCTGCCCGGCGTGGAGGCCGCCGAGC
GCGCCGGCGTGCCCGCCTTCCTGGAGACCAGCGCCCCCCGCAACCTG
CCCTTCTACGAGCGCCTGGGCTTCACCGTGACCGCCGACGTGGAGGT
GCCCGAGGGCCCCCGCACCTGGTGCATGACCCGCAAGCCCGGCGCCT
AATGATCTAGA (SEQ ID NO:91);

hpuro-1:

gctagcgccaccatgaccgagtacaagcctaccgtgcgcctggccactcgcgatgatgtgccccgcgccgtccgc
actctggccgccgctttcgccgactaccccgctacccggcacaccgtggaccccgaccggcacatcgagcgtgtg
acagagttgcaggagctgttcctgacccgcgtcgggctggacatcggcaaggtgtgggtagccgacgacggcgc
ggccgtggccgtgtggactacccccgagagcgttgaggccggcgccgtgttcgccgagatcggcccccgaatgg
ccgagctgagcggcagccgcctggccgcccagcagcaaatggagggcctgcttgccccccatcgtcccaaggag
cccgcctggtttctggccactgtaggagtgagccccgaccaccagggcaagggcttgggcagcgccgtcgtgttg
cccggcgtagaggccgccgaacgcgccggtgtgcccgcctttctggagacaagcgctccgcgtaaccttccattct
acgagcgcctgggcttcaccgtgaccgccgatgtcgaggtgcccgagggaccccggacctggtgcatgactcgc
aagcctggcgcctaatgatctaga (SEQ ID NO:92); and

hpuro-2

GCTAGCGCCACCATGACCGAGTACAAGCCTACCGTGCGCCTGGCCAC
TCGCGATGATGTGCCCCGCGCCGTCCGCACTCTGGCCGCCGCTTTCGC
CGACTACCCCGCTACCCGGCACACCGTGGACCCCGACCGGCACATCG
AGCGTGTGACAGAGTTGCAGGAGCTGTTCCTGACCCGCGTCGGGCTG
GACATCGGCAAGGTGTGGGTAGCCGACGACGGCGCGGCCGTGGCCG
TGTGGACTACCCCCGAGAGCGTTGAGGCCGGCGCCGTGTTCGCCGAG
ATCGGCCCCCGAATGGCCGAGCTGAGCGGCAGCCGCCTGGCCGCCCA
GCAGCAAATGGAGGGCCTGCTTGCCCCCCATCGTCCCAAGGAGCCTG
CCTGGTTTCTGGCCACTGTAGGAGTGAGCCCCGACCACCAGGGCAAG
GGCTTGGGCAGCGCCGTCGTGTTGCCCGGCGTAGAGGCCGCCGAACG
CGCCGGTGTGCCCGCCTTTCTCGAAACAAGCGCACCAAGAAACCTTC
CATTCTACGAGCGCCTGGGCTTCACCGTGACCGCCGATGTCGAGGTG
CCCGAGGGACCTAGGACCTGGTGTATGACACGAAAACCTGGCGCCTA

ATGATCTAGA (SEQ ID NO:93).

The starting puro sequence (from psi STRIKE) has SEQ ID NO:15
(atgaccgagt acaagcccac ggtgcgcctc gccacccgcg acgacgtccc ccgggccgta
cgcaccctcg ccgccgcgtt cgccgactac cccgccacgc gccacaccgt cgacccggac
cgccacatcg agcgggtcac cgagctgcaa gaactcttcc tcacgcgcgt cgggctcgac
atcggcaagg tgtgggtcgc ggacgacggc gccgcggtgg cggtctggac cacgccggag
agcgtcgaag cgggggcggt gttcgccgag atcggcccgc gcatggccga gttgagcggt
tcccggctgg ccgcgcagca acagatggaa ggcctcctgg cgccgcaccg gcccaaggag
cccgcgtggt tcctggccac cgtcggcgtg tcgcccgacc accagggcaa gggtctgggc
agcgccgtcg tgctccccgg agtggaggcg gccgagcgcg ccggggtgcc cgccttcctg
gagacctccg cgccccgcaa cctccccttc tacgagcggc tcggcttcac cgtcaccgcc
gacgtcgagg tgcccgaagg accgcgcacc tggtgcatga cccgcaagcc cggtgcc).

Other synthetic *hyg* and *neo* genes include
hneo-1:
CCACTCAGTGGCCACCATGATCGAGCAGGACGGCCTcCAtGCtGGCAGt
CCCGCaGCCTGGGTcGAGCGCtTGTTCGGgTACGACTGGGCCCAGCAG
ACCATCGGaTGtAGCGAtGCCGCaGTGTTCCGCCTGAGCGCtCAaGGCCG
gCCCGTGCTGTTCGTGAAGACCGACCTGAGCGGCGCCCTGAACGAGC
TtCAaGACGAGGCtGCCCGCCTGAGCTGGCTGGCCACCACCGGtGTaCC
CTGCGCCGCtGTGtTGGAtGTtGTGACCGAaGCCGGCCGCGACTGGCTGC
TGCTGGGCGAGGTGCCtGGCCAGGACCTGCTGAGCAGCCACCTGGCC
CCCGCtGAGAAGGTGAGCATCATGGCCGACGCCATGCGgCGCCTGCAC
ACCCTGGACCCCGCtACaTGCCCCTTCGACCACCAGGCtAAGCACCGC
ATCGAGCGgGCtCGgACCCGCATGGAGGCCGGCCTGGTGGACCAGGAC
GACCTGGACGAGGAGCACCAGGGCCTGGCCCCCGCtGAaCTGTTCGCC
CGCCTGAAaGCCCGCATGCCgGACGGtGAGGACCTGGTtGTGACaCACG
GCGACGCCTGCCTcCCtAACATCATGGTcGAGAACGGgCGCTTCtcCGGC
TTCATCGACTGCGGCCGCCTGGGCGTtGCCGACCGCTACCAGGACATC
GCCCTGGCCACCCGCGACATCGCCGAGGAGCTGGGCGGCGAGTGGG
CCGACCGCTTCCTGGTctTGTACGGCATCGCaGCtCCCGACAGCCAGCG
CATCGCCTTCTACCGCCTGCTGGACGAGTTCTTCTAgTAACCAGgCTCT

GG (SEQ ID NO:38);

hneo-2

CCACTCcGTGGCCACCATGATCGAaCAaGACGGCCTcCAtGCtGGCAGtC
CCGCaGCtTGGGTcGAaCGCtTGTTCGGgTACGACTGGGCCCAGCAGAC
CATCGGaTGtAGCGAtGCgGCCGTGTTCCGtCTaAGCGCtCAaGGCCGgCC
CGTGCTGTTCGTGAAGACCGACCTGAGCGGCGCCCTGAACGAGCTtCA
aGACGAGGCtGCCCGCCTGAGCTGGCTGGCCACCACCGGtGTaCCCTGC
GCCGCtGTGtTGGAtGTtGTGACCGAaGCCGGCCGgGACTGGCTGCTGCT
GGGCGAGGTcCCtGGCCAGGAtCTGCTGAGCAGCCACCTtGCCCCCGCt
GAGAAGGTttcCATCATGGCCGAtGCaATGCGgCGCCTGCACACCCTGG
ACCCCGCtACaTGCCCCTTCGACCACCAGGCtAAGCAtCGgATCGAGCGt
GCtCGgACCCGCATGGAGGCCGGCCTGGTGGACCAGGACGACCTGGA
CGAGGAGCAtCAGGGCCTGGCCCCCGCtGAaCTGTTCGCCCGCCTGAAa
GCCCGCATGCCgGACGGtGAGGACCTGGTtGTGACaCAtGGaGAtGCCTG
CCTcCCtAACATCATGGTcGAGAAtGGcCGCTTCtcCGGCTTCATCGACTG
CGGtCGCCTaGGaGTtGCCGACCGCTACCAGGACATCGCCCTGGCCACC
CGCGACATCGCtGAGGAGCTtGGCGGCGAGTGGGCCGACCGCTTCtTaG
TctTGTACGGCATCGCaGCtCCCGACAGCCAGCGCATCGCCTTCTACCG
CCTGCTcGACGAGTTCTTtTAATGACCAGgCTCTGG (SEQ ID NO:39);

hhyg-1

CCACTCAGTGGCCACCATGAAGAAGCCCGAGCTGACCGCTACCAGCG
TTGAGAAGTTCCTGATCGAGAAGTTCGACAGCGTGAGCGACCTGATG
CAGTTAAGCGAGGGCGAGGAAAGCCGCGCCTTCAGCTTCGATGTCGG
CGGACGCGGCTATGTACTGCGGGTGAATAGCTGCGCTGATGGCTTCT
ACAAAGACCGCTACGTGTACCGCCACTTCGCCAGCGCTGCACTGCCC
ATCCCCGAGGTGCTGGACATCGGCGAGTTCAGCGAGAGCCTGACATA
CTGCATCAGCCGCCGCGCTCAAGGCGTGACTCTCCAAGACCTGCCCG
AGACAGAGCTGCCCGCTGTGCTACAGCCTGTCGCCGAGGCTATGGAC
GCTATTGCCGCCGCCGACCTGAGCCAGACCAGCGGCTTCGGCCCATT
CGGGCCCCAAGGCATCGGCCAGTACACCACCTGGCGCGACTTCATCT
GCGCCATTGCTGATCCCCATGTCTACCACTGGCAGACCGTGATGGAC
GACACCGTGAGCGCCAGCGTAGCTCAAGCCCTGGACGAGCTGATGCT

GTGGGCCGAGGACTGCCCCGAGGTGCGCCATCTCGTCCATGCCGACT
TCGGCAGCAACAACGTCCTGACCGACAACGGCCGCATCACCGCCGTA
ATCGACTGGAGCGAGGCCATGTTCGGGGACAGTCAGTACGAGGTGGC
CAACATCTTCTTCTGGCGGCCCTGGCTGGCCTGCATGGAGCAGCAAA
CCCGCTACTTCGAGCGCCGCCATCCCGAGCTGGCCGGCAGCCCCCGT
CTGCGAGCCTACATGCTGCGCATCGGCCTGGATCAGCTCTACCAGAG
CCTCGTGGACGGCAACTTCGACGATGCTGCCTGGGCTCAAGGCCGCT
GCGATGCCATCGTCCGCAGCGGGGCCGGCACCGTCGGTCGCACACAA
ATCGCTCGCCGGAGCGCCGCCGTATGGACCGACGGCTGCGTCGAGGT
GCTGGCCGACAGCGGCAACCGCCGGCCCAGTACACGACCGCGCGCTA
AGGAGTAGTAACCAGCTCTTGG (SEQ ID NO:30);

hhyg-2:

CCACTCCGTGGCCACCATGAAGAAGCCCGAGCTGACCGCTACCAGCG
TTGAAAAATTTCTCATCGAGAAGTTCGACAGTGTGAGCGACCTGATG
CAGTTGTCGGAGGGCGAAGAGAGCCGAGCCTTCAGCTTCGATGTCGG
CGGACGCGGCTATGTACTGCGGGTGAATAGCTGCGCTGATGGCTTCT
ACAAAGACCGCTACGTGTACCGCCACTTCGCCAGCGCTGCACTACCC
ATCCCCGAAGTGTTGGACATCGGCGAGTTCAGCGAGAGCCTGACATA
CTGCATCAGTAGACGCGCCCAAGGCGTTACTCTCCAAGACCTCCCCG
AAACAGAGCTGCCTGCTGTGTTACAGCCTGTCGCCGAAGCTATGGAT
GCTATTGCCGCCGCCGACCTCAGTCAAACCAGCGGCTTCGGCCCATT
CGGGCCCCAAGGCATCGGCCAGTACACAACCTGGCGGGATTTCATTT
GCGCCATTGCTGATCCCCATGTCTACCACTGGCAGACCGTGATGGAC
GACACCGTGTCCGCCAGCGTAGCTCAAGCCCTGGACGAACTGATGCT
GTGGGCCGAAGACTGTCCCGAGGTGCGCCACCTCGTCCATGCCGACT
TCGGCAGCAACAACGTCCTGACCGACAACGGCCGCATCACCGCCGTA
ATCGACTGGAGCGAGGCTATGTTCGGGGACAGTCAGTACGAGGTGGC
CAACATCTTCTTCTGGCGGCCCTGGCTGGCTTGCATGGAGCAGCAGA
CTCGCTACTTCGAGCGCCGGCATCCCGAGCTGGCCGGCAGCCCTCGT
CTGCGAGCCTACATGCTGCGCATCGGCCTGGATCAGCTCTACCAGAG
CCTCGTGGACGGCAACTTCGACGATGCTGCCTGGGCTCAAGGCCGCT
GCGATGCCATCGTCCGCAGCGGGGCCGGCACCGTCGGTCGCACACAA

ATCGCTCGCCGGAGCGCCGCCGTATGGACCGACGGCTGCGTCGAGGT
GCTGGCCGACAGCGGCAACCGCCGGCCCAGTACACGACCGCGCGCTA
AGGAGTAGTAACCAGCTCTTGG (SEQ ID NO:42);

hHygro (*Sac*I site in ORF near 5′ end, insert in-frame linker coding for 12 amino acids at 3′ end, and *Sna*BI site added at 3′ end in ORF)
aagcttgctagcgccaccatgaagaagcccgagctcaccgctaccagcgttgaaaaatttctcatcgagaagttcga
cagtgtgagcgacctgatgcagttgtcggagggcgaagagagccgagccttcagcttcgatgtcggcggacgcgg
ctatgtactgcgggtgaatagctgcgctgatggcttctacaaagaccgctacgtgtaccgccacttcgccagcgctgc
actacccatccccgaagtgttggacatcggcgagttcagcgagagcctgacatactgcatcagtagacgcgcccaa
ggcgttactctccaagacctccccgaaacagagctgcctgctgtgttacagcctgtcgccgaagctatggatgctatt
gccgccgccgacctcagtcaaaccagcggcttcggcccattcgggccccaaggcatcggccagtacacaacctg
gcgggatttcatttgcgccattgctgatccccatgtctaccactggcagaccgtgatggacgacaccgtgtccgccag
cgtagctcaagccctggacgaactgatgctgtgggccgaagactgtcccgaggtgcgccacctcgtccatgccgac
ttcggcagcaacaacgtcctgaccgacaacggccgcatcaccgccgtaatcgactggtccgaagctatgttcgggg
acagtcagtacgaggtggccaacatcttcttctggcggccctggctggcttgcatggagcagcagactcgctacttc
gagcgccggcatcccgagctggccggcagccctcgtctgcgagcctacatgctgcgcatcggcctggatcagctc
taccagagcctcgtggacggcaacttcgacgatgctgcctgggctcaaggccgctgcgatgccatcgtccgcagc
ggggccggcaccgtcggtcgcacacaaatcgctcgccggagcgccgccgtatggaccgacggctgcgtcgaggt
gctggccgacagcggcaaccgccggcccagtacacgaccgcgcgctaaggagggtggcggagggagcggtgg
cggaggttcctacgtatagtctagactcgag (SEQ ID NO:70);

hhyg-4
atgaagaagcccgagctcaccgctaccagcgttgaaaaatttctcatcgagaagttcgacagtgtgagcgacctgat
gcagttgtcggagggcgaagagagccgagccttcagcttcgatgtcggcggacgcggctatgtactgcgggtgaa
tagctgcgctgatggcttctacaaagaccgctacgtgtaccgccacttcgccagcgctgcactacccatccccgaag
tgttggacatcggcgagttcagcgagagcctgacatactgcatcagtagacgcgcccaaggcgttactctccaaga
cctccccgaaacagagctgcctgctgtgttacagcctgtcgccgaagctatggatgctattgccgccgccgacctca
gtcaaaccagcggcttcggcccattcgggccccaaggcatcggccagtacacaacctggcgggatttcatttgcgc
cattgctgatccccatgtctaccactggcagaccgtgatggacgacaccgtgtccgccagcgtagctcaagccctgg
acgaactgatgctgtgggccgaagactgtcccgaggtgcgccacctcgtccatgccgacttcggcagcaacaacgt
cctgaccgacaacggccgcatcaccgccgtaatcgactggtccgaagctatgttcgggacagtcagtacgaggtg
gccaacatcttcttctggcggccctggctggcttgcatggagcagcagactcgctacttcgagcgccggcatcccga

gctggccggcagccctcgtctgcgagcctacatgctgcgcatcggcctggatcagctctaccagagcctcgtggac
ggcaacttcgacgatgctgcctgggctcaaggccgctgcgatgccatcgtccgcagcggggccggcaccgtcggt
cgcacacaaatcgctcgccggagcgcagccgtatggaccgacggctgcgtcgaggtgctggccgacagcggca
accgccggcccagtacacgaccgcgcgctaaggaaggcggtggaggtagtggtggcggaggtagctacgta
(SEQ ID NO:71);

hneo-4:
GCTAGCGCCACCATGATCGAACAAGACGGCCTCCATGCTGGCAGTCC
CGCAGCTTGGGTCGAACGCTTGTTCGGGTACGACTGGGCCCAGCAGA
CCATCGGATGTAGCGATGCGGCCGTGTTCCGTCTAAGCGCTCAAGGC
CGGCCCGTGCTGTTCGTGAAGACCGACCTGAGCGGCGCCCTGAACGA
GCTTCAAGACGAGGCTGCCCGCCTGAGCTGGCTGGCCACCACCGGTG
TACCCTGCGCCGCTGTGTTGGATGTTGTGACCGAAGCCGGCCGGGAC
TGGCTGCTGCTGGGCGAGGTCCCTGGCCAGGATCTGCTGAGCAGCCA
CCTTGCCCCCGCTGAGAAGGTTTCCATCATGGCCGATGCAATGCGGC
GCCTGCACACCCTGGACCCCGCTACATGCCCCTTCGACCACCAGGCT
AAGCATCGGATCGAGCGTGCTCGGACCCGCATGGAGGCCGGCCTGGT
GGACCAGGACGACCTGGACGAGGAGCATCAGGGCCTGGCCCCCGCT
GAACTGTTCGCCCGCCTGAAAGCCCGCATGCCGGACGGTGAGGACCT
GGTTGTGACACATGGTGATGCCTGCCTCCCTAACATCATGGTCGAGA
ATGGCCGCTTCTCCGGCTTCATCGACTGCGGTCGCCTAGGAGTTGCCG
ACCGCTACCAGGACATCGCCCTGGCCACCCGCGACATCGCTGAGGAG
CTTGGCGGCGAGTGGGCCGACCGCTTCTTAGTCTTGTACGGCATCGC
AGCTCCCGACAGCCAGCGCATCGCCTTCTACCGCCTGCTCGACGAGT
TCTTTTAATCTAGA
(SEQ ID NO:72);
and
hneo-5:
GCTAGCGCCACCATGATCGAACAAGACGGCCTCCATGCTGGCAGTCC
CGCAGCTTGGGTCGAACGCTTGTTCGGGTACGACTGGGCCCAGCAGA
CCATCGGATGTAGCGATGCGGCCGTGTTCCGTCTAAGCGCTCAAGGC
CGGCCCGTGCTGTTCGTGAAGACCGACCTGAGCGGCGCCCTGAACGA
GCTTCAAGACGAGGCTGCCCGCCTGAGCTGGCTGGCCACCACCGGCG

TACCCTGCGCCGCTGTGTTGGATGTTGTGACCGAAGCCGGCCGGGAC
TGGCTGCTGCTGGGCGAGGTCCCTGGCCAGGATCTGCTGAGCAGCCA
CCTTGCCCCCGCTGAGAAGGTTTCTATCATGGCCGATGCAATGCGGC
GCCTGCACACCCTGGACCCCGCTACCTGCCCCTTCGACCACCAGGCT
AAGCATCGGATCGAGCGTGCTCGGACCCGCATGGAGGCCGGCCTGGT
GGACCAGGACGACCTGGACGAGGAGCATCAGGGCCTGGCCCCCGCT
GAACTGTTCGCCCGACTGAAAGCCCGCATGCCGGACGGTGAGGACCT
GGTTGTCACACACGGAGATGCCTGCCTCCCTAACATCATGGTCGAGA
ATGGCCGCTTCTCCGGCTTCATCGACTGCGGTCGCCTAGGAGTTGCCG
ACCGCTACCAGGACATCGCCCTGGCCACCCGCGACATCGCTGAGGAG
CTTGGCGGCGAGTGGGCCGACCGCTTCTTAGTCTTGTACGGCATCGC
AGCTCCCGACAGCCAGCGCATCGCCTTCTACCGCTTGCTCGACGAGTT
CTTTTAATGATCTAGA(SEQ ID NO:73).

The synthetic nucleotide sequence of the invention may be employed in fusion constructs. For instance, a synthetic sequence for a selectable polypeptide may be fused to a wild-type sequence or to another synthetic sequence which encodes a different polypeptide. For instance, the *neo* sequence in the following examples of a synthetic *Renilla* luciferase-*neo* sequence may be replaced with a synthetic *neo* sequence of the invention:

atggcttccaaggtgtacgaccccgagcaacgcaaacgcatgatcactgggcctcagtggtgggctcgctgcaagc
aaatgaacgtgctggactccttcatcaactactatgattccgagaagcacgccgagaacgccgtgatttttctgcatgg
taacgctgcctccagctacctgtggaggcacgtcgtgcctcacatcgagcccgtggctagatgcatcatccctgatct
gatcggaatgggtaagtccggcaagagcgggaatggctcatatcgcctcctggatcactacaagtacctcaccgctt
ggttcgagctgctgaaccttccaaagaaaatcatctttgtgggccacgactgggggggcttgtctggcctttcactactc
ctacgagcaccaagacaagatcaaggccatcgtccatgctgagagtgtcgtggacgtgatcgagtcctgggacga
gtggcctgacatcgaggaggatatcgccctgatcaagagcgaagagggcgagaaaatggtgcttgagaataacttc
ttcgtcgagaccatgctcccaagcaagatcatgcggaaactggagcctgaggagttcgctgcctacctggagccatt
caaggagaagggcgaggttagacggcctaccctctcctggcctcgcgagatccctctcgttaagggaggcaagcc
cgacgtcgtccagattgtccgcaactacaacgcctaccttcgggccagcgacgatctgcctaagatgttcatcgagtc
cgaccctgggttcttttccaacgctattgtcgagggagctaagaagttccctaacaccgagttcgtgaaggtgaaggg
cctccacttcagccaggaggacgctccagatgaaatgggtaagtacatcaagagcttcgtggagcgcgtgctgaag
aacgagcagaccggtggtgggagcggaggtggcggatcaggtggcggaggctccggagggattgaacaagatg

gattgcacgcaggttctccggccgcttgggtggagaggctattcggctatgactgggcacaacagacaatcggctg
ctctgatgccgccgtgttccggctgtcagcgcaggggcgcccggttctttttgtcaagaccgacctgtccggtgccct
gaatgaactgcaggacgaggcagcgcggctatcgtggctggccacgacgggcgttccttgcgcagctgtgctcga
cgttgtcactgaagcgggaagggactggctgctattgggcgaagtgccggggcaggatctcctgtcatctcaccttg
ctcctgccgagaaagtatccatcatggctgatgcaatgcggcggctgcatacgcttgatccggctacctgcccattcg
accaccaagcgaaacatcgcatcgagcgagcacgtactcggatggaagccggtcttgtcgatcaggatgatctgga
cgaagagcatcaggggctcgcgccagccgaactgttcgccaggctcaaggcgcgcatgcccgacggcgaggat
ctcgtcgtgacccatggcgatgcctgcttgccgaatatcatggtggaaaatggccgcttttctggattcatcgactgtg
gccggctgggtgtggcggaccgctatcaggacatagcgttggctacccgtgatattgctgaagagcttggcggcga
atgggctgaccgcttcctcgtgctttacggtatcgccgctcccgattcgcagcgcatcgccttctatcgccttcttgacg
agttcttctaa (hrl-neo fusion; SEQ ID NO:12)

and

atgattgaacaagatggattgcacgcaggttctccggccgcttgggtggagaggctattcggctatgactgggcaca
acagacaatcggctgctctgatgccgccgtgttccggctgtcagcgcaggggcgcccggttctttttgtcaagaccg
acctgtccggtgccctgaatgaactgcaggacgaggcagcgcggctatcgtggctggccacgacgggcgttcctt
gcgcagctgtgctcgacgttgtcactgaagcgggaagggactggctgctattgggcgaagtgccggggcaggatc
tcctgtcatctcaccttgctcctgccgagaaagtatccatcatggctgatgcaatgcggcggctgcatacgcttgatcc
ggctacctgcccattcgaccaccaagcgaaacatcgcatcgagcgagcacgtactcggatggaagccggtcttgtc
gatcaggatgatctggacgaagagcatcaggggctcgcgccagccgaactgttcgccaggctcaaggcgcgcat
gcccgacggcgaggatctcgtcgtgacccatggcgatgcctgcttgccgaatatcatggtggaaaatggccgctttt
ctggattcatcgactgtggccggctgggtgtggcggaccgctatcaggacatagcgttggctacccgtgatattgctg
aagagcttggcggcgaatgggctgaccgcttcctcgtgctttacggtatcgccgctcccgattcgcagcgcatcgcc
ttctatcgccttcttgacgagttcttcaccggtggtgggagcggaggtggcggatcaggtggcggaggctccggag
gggcttccaaggtgtacgaccccgagcaacgcaaacgcatgatcactgggcctcagtggtgggctcgctgcaagc
aaatgaacgtgctggactccttcatcaactactatgattccgagaagcacgccgagaacgccgtgatttttctgcatgg
taacgctgcctccagctacctgtggaggcacgtcgtgcctcacatcgagcccgtggctagatgcatcatccctgatct
gatcggaatgggtaagtccggcaagagcgggaatggctcatatcgcctcctggatcactacaagtacctcaccgctt
ggttcgagctgctgaaccttccaaagaaaatcatctttgtgggccacgactgggggcttgtctggcctttcactactc
ctacgagcaccaagacaagatcaaggccatcgtccatgctgagagtgtcgtggacgtgatcgagtcctgggacga
gtggcctgacatcgaggaggatatcgccctgatcaagagcgaagagggcgagaaaatggtgcttgagaataacttc
ttcgtcgagaccatgctcccaagcaagatcatgcggaaactggagcctgaggagttcgctgcctacctggagccatt
caaggagaagggcgaggttagacggcctaccctctcctggcctcgcgagatccctctcgttaagggaggcaagcc
cgacgtcgtccagattgtccgcaactacaacgcctaccttcgggccagcgacgatctgcctaagatgttcatcgagtc

cgaccctgggttcttttccaacgctattgtcgagggagctaagaagttccctaacaccgagttcgtgaaggtgaaggg cctccacttcagccaggaggacgctccagatgaaatgggtaagtacatcaagagcttcgtggagcgcgtgctgaag aacgagcagtaa (neo-hrl-fusion; SEQ ID NO:13).

Example 5

Transcription Factor Binding Sites Used to Identify Sites in Selected Synthetic Sequences

TF binding site libraries

The TF binding site library ("Matrix Family Library") is part of the GEMS Launcher package. Table 16 shows the version of the Matrix Family Library which was used in the design of a particular sequence and Table 17 shows a list of all vertebrate TF binding sites ("matrices") in Matrix Family Library Version 2.4, as well as all changes made to vertebrate matrices in later versions up to 4.1 (section "GENOMATIX MATRIX FAMILY LIBRARY INFORMATION Versions 2.4 to 4.1"). (Genomatix has a copyright to all Matrix Library Family information).

Table 16

Synthetic DNA sequence	**Genomatix Matrix Family Library**
pGL4B-NN3*	Version 2.4 May 2002
luc2A8 and luc2B10	Version 3.0 Nov 2002 Version 3.1.1 April 2003
hhyg3 hneo3	Version 3.1.2 June 2003
hhyg4	Version 3.3 August 2003
*Spe*I-*Nco*I-Ver2 **	Version 4.0 Nov 2003
hneo5 hpuro2	Version 4.1 Feb 2004

*_Not_I-_Nco_I fragment in pGL4 including _amp_ gene (pGL4B-NN3)

**_Spe_I-_Nco_I-Ver2 (replacement for _Spe_I-_Nco_I fragment in pGL4B-NN3

Table 17

GENOMATIX MATRIX FAMILY LIBRARY INFORMATION
Versions 2.4 to 4.1

A. Matrix Family Library Version 2.4

Matrix Family Library Version 2.4 (May 2002) contains 412 weight matrices in 193 families
(Vertebrates: 275 matrices in 106 families)
Vertebrates

Family	Family Information	Matrix Name	Information
V$AHRR	AHR-arnt heterodimers and AHR-related factors	V$AHRARNT.01	aryl hydrocarbon receptor / Arnt heterodimers
		V$AHR.01	aryl hydrocarbon / dioxin receptor
		V$AHRARNT.02	aryl hydrocarbon / Arnt heterodimers, fixed core
V$AP1F	AP1 and related factors	V$AP1.01	AP1 binding site
		V$AP1.02	activator protein 1
		V$AP1.03	activator protein 1
		V$AP1FJ.01	activator protein 1
		V$NFE2.01	NF-E2 p45
		V$VMAF.01	v-Maf
		V$TCF11MAFG.01	TCF11/MafG heterodimers, binding to subclass of AP1 sites
		V$BEL1.01	Bel-1 similar region
V$AP2F	Activator Protein 2	V$AP2.01	activator protein 2
V$AP4R	AP4 and Related proteins	V$AP4.01	activator protein 4
		V$AP4.02	activator protein 4
		V$TH1E47.01	Thing1/E47 heterodimer, TH1 bHLH member specific expression in a variety of embryonic tissues

Family	Family Information	Matrix Name	Information
		V$TAL1ALPHAE47.01	Tal-1alpha/E47 heterodimer
		V$TAL1BETAE47.01	Tal-1beta/E47 heterodimer
		V$TAL1BETAITF2.01	Tal-1beta/ITF-2 heterodimer
		V$AP4.03	activator protein 4
V$AREB	Atp1a1 regulatory element binding	V$AREB6.04	AREB6 (Atp1a1 regulatory element binding factor 6)
		V$AREB6.02	AREB6 (Atp1a1 regulatory element binding factor 6)
		V$AREB6.03	AREB6 (Atp1a1 regulatory element binding factor 6)
		V$AREB6.01	AREB6 (Atp1a1 regulatory element binding factor 6)
V$ARP1	Apolipoprotein aI and cIII gene Repressor Protein	V$ARP1.01	apolipoprotein AI regulatory protein 1
V$BARB	BARbiturate-Inducible El. box from Pro+eukaryot. genes	V$BARBIE.01	barbiturate-inducible element
V$BCL6	POZ domain zinc finger expressed in B-Cells	V$BCL6.01	POZ/zinc finger protein, transcriptional repressor, translocations observed in diffuse large cell lymphoma
		V$BCL6.02	POZ/zinc finger protein, transcriptional repressor, translocations observed in diffuse large cell lymphoma
V$BRAC	Brachyury gene, mesoderm	V$TBX5.01	T-Box factor 5 site (TBX5), mutations related to Holt-Oram

Family	Family Information	Matrix Name	Information
	developmental factor		syndrome
		V$BRACH.01	Brachyury
V$BRNF	Brn POU domain factors	V$BRN3.01	POU transcription factor Brn-3
		V$BRN2.01	POU factor Brn-2 (N-Oct 3)
V$CABL	C-abl DNA binding sites	V$CABL.01	Multifunctional c-Abl src type tyrosine kinase
V$CART	Cart-1 (cartilage homeoprotein 1)	V$XVENT2.01	Xenopus homeodomain factor Xvent-2; early BMP signaling response
		V$CART1.01	Cart-1 (cartilage homeoprotein 1)
V$CDXF	Vertebrate caudal related homeodomain protein	V$CDX2.01	Cdx-2 mammalian caudal related intestinal transcr. factor
V$CEBP	Ccaat/Enhancer Binding Protein	V$CEBPB.01	CCAAT/enhancer binding protein beta
		V$CEBP.02	C/EBP binding site
V$CHOP	CHOP binding protein	V$CHOP.01	heterodimers of CHOP and C/EBPalpha
V$CLOX	CLOX and CLOX homology (CDP) factors	V$CDPCR3HD.01	cut-like homeodomain protein
		V$CDP.01	cut-like homeodomain protein
		V$CDP.02	transcriptional repressor CDP
		V$CDPCR3.01	cut-like homeodomain protein
		V$CLOX.01	Clox
V$CMYB	C-MYB, cellular transcriptional activator	V$CMYB.01	c-Myb, important in hematopoesis, cellular equivalent to avian myoblastosis virus

Family	Family Information	Matrix Name	Information
			oncogene v-myb
V$COMP	factors which COoperate with Myogenic Proteins	V$COMP1.01	COMP1, cooperates with myogenic proteins in multicomponent complex
V$COUP	Repr. of RXR-mediated activ. & retinoic acid responses	V$COUP.01	COUP antagonizes HNF-4 by binding site competition or synergizes by direct protein - protein interaction with HNF-4
V$CP2F	CP2-erythrocyte Factor related to drosophila Elf1	V$CP2.01	CP2
V$CREB	Camp-Responsive Element Binding proteins	V$CREBP1.01	cAMP-responsive element binding protein 1
		V$CREBP1CJUN.01	CRE-binding protein 1/c-Jun heterodimer
		V$CREB.01	cAMP-responsive element binding protein
		V$HLF.01	hepatic leukemia factor
		V$E4BP4.01	E4BP4, bZIP domain, transcriptional repressor
		V$CREB.02	cAMP-responsive element binding protein
		V$CREB.03	cAMP-response element-binding protein
		V$CREB.04	cAMP-response element binding protein
		V$CREBP1.02	CRE-binding protein 1
		V$ATF.02	ATF binding site
		V$ATF.01	activating transcription factor
		V$TAXCREB.01	Tax/CREB complex
		V$TAXCREB.02	Tax/CREB complex

Family	Family Information	Matrix Name	Information
		V$VJUN.01	v-Jun
V$E2FF	E2F-myc activator/cell cycle regulator	V$E2F.02	E2F, involved in cell cycle regulation, interacts with Rb p107 protein
		V$E2F.03	E2F, involved in cell cycle regulation, interacts with Rb p107 protein
		V$E2F.01	E2F, involved in cell cycle regulation, interacts with Rb p107 protein
V$E2TF	papillioma virus E2 Transcriptional activator	V$E2.01	BPV bovine papilloma virus regulator E2
		V$E2.02	papilloma virus regulator E2
V$EBOR	E-BOx Related factors	V$DELTAEF1.01	deltaEF1
		V$XBP1.01	X-box-binding protein 1
V$EBOX	E-BOX binding factors	V$USF.02	upstream stimulating factor
		V$USF.03	upstream stimulating factor
		V$MYCMAX.03	MYC-MAX binding sites
		V$SREBP.03	Sterol regulatory element binding protein
		V$SREBP.02	Sterol regulatory element binding protein
		V$MYCMAX.02	c-Myc/Max hetero dimer
		V$NMYC.01	N-Myc
		V$ATF6.01	Member of b-zip family, induced by ER damage/stress
		V$USF.01	upstream stimulating factor

Family	Family Information	Matrix Name	Information
		V$MYCMAX.01	c-Myc/Max heterodimer
		V$MAX.01	Max
		V$ARNT.01	AhR nuclear translocator homodimers
		V$SREBP.01	Sterol regulatory element binding protein 1 and 2
V$ECAT	Enhancer-CcAaT binding factors	V$NFY.02	nuclear factor Y (Y-box binding factor)
		V$NFY.03	nuclear factor Y (Y-box binding factor)
		V$NFY.01	nuclear factor Y (Y-box binding factor)
V$EGRF	EGR/nerve growth Factor Induced protein C & rel. fact.	V$EGR1.01	Egr-1/Krox-24/NGFI-A immediate-early gene product
		V$EGR2.01	Egr-2/Krox-20 early growth response gene product
		V$EGR3.01	early growth response gene 3 product
		V$NGFIC.01	nerve growth factor-induced protein C
		V$WT1.01	Wilms Tumor Suppressor
V$EKLF	Erythroid krueppel like factor	V$EKLF.01	Erythroid krueppel like factor (EKLF)
V$ETSF	Human and murine ETS1 Factors	V$CETS1P54.01	c-Ets-1(p54)
		V$NRF2.01	nuclear respiratory factor 2
		V$GABP.01	GABP: GA binding protein
		V$ELK1.02	Elk-1

Family	Family Information	Matrix Name	Information
		V$FLI.01	ETS family member FLI
		V$ETS2.01	c-Ets-2 binding site
		V$ETS1.01	c-Ets-1 binding site
		V$ELK1.01	Elk-1
		V$PU1.01	Pu.1 (Pu120) Ets-like transcription factor identified in lymphoid B-cells
V$EVI1	EVI1-myleoid transforming protein	V$EVI1.06	Ecotropic viral integration site 1 encoded factor
		V$EVI1.02	Ecotropic viral integration site 1 encoded factor
		V$EVI1.03	Ecotropic viral integration site 1 encoded factor
		V$EVI1.05	Ecotropic viral integration site 1 encoded factor
		V$EVI1.04	Ecotropic viral integration site 1 encoded factor
		V$EVI1.01	Ecotropic viral integration site 1 encoded factor
V$FKHD	Fork Head Domain factors	V$HFH1.01	HNF-3/Fkh Homolog 1
		V$HFH2.01	HNF-3/Fkh Homolog 2
		V$HFH3.01	HNF-3/Fkh Homolog 3 (= Freac-6)
		V$HFH8.01	HNF-3/Fkh Homolog-8
		V$XFD1.01	Xenopus fork head domain factor 1

Family	Family Information	Matrix Name	Information
		V$XFD2.01	Xenopus fork head domain factor 2
		V$XFD3.01	Xenopus fork head domain factor 3
		V$HNF3B.01	Hepatocyte Nuclear Factor 3beta
		V$FREAC2.01	Fork head RElated ACtivator-2
		V$FREAC3.01	Fork head RElated ACtivator-3
		V$FREAC4.01	Fork head RElated ACtivator-4
		V$FREAC7.01	Fork head RElated ACtivator-7
V$GATA	GATA binding factors	V$LMO2COM.02	complex of Lmo2 bound to Tal-1, E2A proteins, and GATA-1, half-site 2
		V$GATA1.04	GATA-binding factor 1
		V$GATA1.05	GATA-binding factor 1
		V$GATA2.01	GATA-binding factor 2
		V$GATA2.02	GATA-binding factor 2
		V$GATA3.01	GATA-binding factor 3
		V$GATA3.02	GATA-binding factor 3
		V$GATA.01	GATA binding site (consensus)
		V$GATA1.03	GATA-binding factor 1
		V$GATA1.01	GATA-binding factor 1
		V$GATA1.02	GATA-binding factor 1
V$GFI1	Growth Factor Independence-transcriptional	V$GFI1.01	growth factor independence 1 zinc finger protein acts as

Family	Family Information	Matrix Name	Information
	repressor		transcriptional repressor
V$GKLF	Gut-enriched Krueppel Like binding Factor	V$GKLF.01	gut-enriched Krueppel-like factor
V$GREF	Glucocorticoid responsive and related elements	V$GRE.01	Glucocorticoid receptor, C2C2 zinc finger protein binds glucocorticoid dependent to GREs
		V$ARE.01	Androgene receptor binding site
		V$PRE.01	Progesterone receptor binding site
V$HAML	Human Acute Myelogenous Leukemia factors	V$AML1.01	runt-factor AML-1
V$HEAT	HEAT shock factors	V$HSF1.01	heat shock factor 1
V$HEN1	E-box binding factor without transcript. activation	V$HEN1.01	HEN1
		V$HEN1.02	HEN1
V$HMTB	Human muscle-specific Mt binding site	V$MTBF.01	muscle-specific Mt binding site
V$HNF1	Hepatic Nuclear Factor 1	V$HNF1.01	hepatic nuclear factor 1
		V$HNF1.02	Hepatic nuclear factor 1
V$HNF4	Hepatic Nuclear Factor 4	V$HNF4.01	Hepatic nuclear factor 4
		V$HNF4.02	Hepatic nuclear factor 4
V$HOMS	Homeodomain subfamily S8	V$S8.01	Binding site for S8 type homeodomains
V$HOXF	Factors with moderate activity to homeo domain consensus sequence	V$HOXA9.01	Member of the vertebrate HOX - cluster of homeobox factors
		V$HOX1-3.01	Hox-1.3, vertebrate homeobox protein
V$IKRS	Ikaros zinc finger family	V$LYF1.01	LyF-1 (Ikaros 1), enriched in B and T

Family	Family Information	Matrix Name	Information
			lymphocytes
		V$IK2.01	Ikaros 2, potential regulator of lymphocyte differentiation
		V$IK1.01	Ikaros 1, potential regulator of lymphocyte differentiation
		V$IK3.01	Ikaros 3, potential regulator of lymphocyte differentiation
V$IRFF	Interferon Regulatory Factors	V$IRF1.01	interferon regulatory factor 1
		V$IRF2.01	interferon regulatory factor 2
		V$ISRE.01	interferon-stimulated response element
V$LEFF	LEF1/TCF	V$LEF1.01	TCF/LEF-1, involved in the Wnt signal transduction pathway
V$LTUP	Lentiviral Tata UPstream element	V$TAACC.01	Lentiviral TATA upstream element
V$MEF2	MEF2-myocyte-specific enhancer-binding factor	V$MEF2.05	MEF2
		V$MEF2.01	myogenic enhancer factor 2
		V$HMEF2.01	myocyte enhancer factor
		V$MMEF2.01	myocyte enhancer factor
		V$RSRFC4.01	related to serum response factor, C4
		V$RSRFC4.02	related to serum response factor, C4
		V$AMEF2.01	myocyte enhancer factor
		V$MEF2.02	myogenic MADS factor MEF-2

Family	Family Information	Matrix Name	Information
		V$MEF2.03	myogenic MADS factor MEF-2
		V$MEF2.04	myogenic MADS factor MEF-2
V$MEF3	MEF3 BINDING SITES	V$MEF3.01	MEF3 binding site, present in skeletal muscle-specific transcriptional enhancers
V$MEIS	Homeodomain factor aberrantly expressed in myeloid leukemia	V$MEIS1.01	Homeobox protein MEIS1 binding site
V$MINI	Muscle INItiator	V$MUSCLE_INI.01	Muscle Initiator Sequence
		V$MUSCLE_INI.02	Muscle Initiator Sequence
		V$MUSCLE_INI.03	Muscle Initiator Sequence
V$MOKF	Mouse Krueppel like factor	V$MOK2.01	Ribonucleoprotein associated zinc finger protein MOK-2
V$MTF1	Metal induced transcription factor	V$MTF-1.01	Metal transcription factor 1, MRE
V$MYOD	MYOblast Determining factor	V$MYOD.02	myoblast determining factor
		V$MYF5.01	Myf5 myogenic bHLH protein
		V$MYOD.01	myoblast determination gene product
		V$LMO2COM.01	complex of Lmo2 bound to Tal-1, E2A proteins, and GATA-1, half-site 1
		V$E47.01	MyoD/E47 and MyoD/E12 dimers
		V$E47.02	TAL1/E47 dimers

Family	Family Information	Matrix Name	Information
V$MYOF	MYOgenic Factors	V$NF1.01	nuclear factor 1
		V$MYOGNF1.01	myogenin / nuclear factor 1 or related factors
V$MYT1	Xenopus MYT1 C2HC zinc finger protein	V$MYT1.02	MyT1 zinc finger transcription factor involved in primary neurogenesis
		V$MYT1.01	MyT1 zinc finger transcription factor involved in primary neurogenesis
V$MZF1	Myeloid Zinc Finger 1 factors	V$MZF1.01	MZF1
V$NFAT	Nuclear Factor of Activated T-cells	V$NFAT.01	Nuclear factor of activated T-cells
V$NFKB	Nuclear Factor Kappa B/c-rel	V$CREL.01	c-Rel
		V$NFKAPPAB.01	NF-kappaB
		V$NFKAPPAB65.01	NF-kappaB (p65)
		V$NFKAPPAB50.01	NF-kappaB (p50)
		V$NFKAPPAB.02	NF-kappaB
		V$NFKAPPAB.03	NF-kappaB
V$NKXH	NKX - Homeodomain sites	V$NKX25.01	homeo domain factor Nkx-2.5/Csx, tinman homolog, high affinity sites
		V$NKX25.02	homeo domain factor Nkx-2.5/Csx, tinman homolog low affinity sites
		V$NKX31.01	prostate-specific homeodomain protein NKX3.1
V$NOLF	Neuron-specific-OLFactory factor	V$OLF1.01	olfactory neuron-specific factor

Family	Family Information	Matrix Name	Information
V$NRSF	Neuron-Restrictive Silencer Factor	V$NRSF.01	neuron-restrictive silencer factor
		V$NRSE.01	neural-restrictive-silencer-element
V$OAZF	Olfactory associated zinc finger protein	V$ROAZ.01	Rat C2H2 Zn finger protein involved in olfactory neuronal differentiation
V$OCT1	OCTamer binding protein	V$OCT1.02	octamer-binding factor 1
		V$OCT1.06	octamer-binding factor 1
		V$OCT.01	Octamer binding site (OCT1/OCT2 consensus)
		V$OCT1.05	octamer-binding factor 1
		V$OCT1.04	octamer-binding factor 1
		V$OCT1.03	octamer-binding factor 1
		V$OCT1.01	octamer-binding factor 1
V$OCTB	OCT6 Binding factors_astrocytes + glioblastoma cells	V$TST1.01	POU-factor Tst-1/Oct-6
V$OCTP	OCT1 binding factor (POU-specific domain)	V$OCT1P.01	octamer-binding factor 1, POU-specific domain
V$P53F	p53 tumor suppr.-neg. regulat. of the tumor suppr. Rb	V$P53.01	tumor suppressor p53
V$PAX1	PAX-1 binding site	V$PAX1.01	Pax1 paired domain protein, expressed in the developing vertebral column of mouse embryos
V$PAX3	PAX-3 binding sites	V$PAX3.01	Pax-3 paired domain protein, expressed in embryogenesis, mutations correlate to Waardenburg Syndrome

Family	Family Information	Matrix Name	Information
V$PAX4	Heterogeneous PAX-4 binding sites	V$PAX4.01	Pax-4 paired domain protein, together with PAX-6 involved in pancreatic development
V$PAX5	PAX-5 / PAX-9 B-cell-specific activating protein	V$PAX9.01	zebrafish PAX9 binding sites
		V$PAX5.01	B-cell-specific activating protein
		V$PAX5.02	B-cell-specific activating protein
V$PAX6	Activ. involved in Iris development in the mouse eye	V$PAX6.01	Pax-6 paired domain protein
V$PAX8	PAX-2/5/8 binding sites	V$PAX8.01	PAX 2/5/8 binding site
V$PBXF	Homeo domain factor PBX-1	V$PBX1.01	homeo domain factor Pbx-1
V$PCAT	Promoter-CcAaT binding factors	V$ACAAT.01	Avian C-type LTR CCAAT box
		V$CAAT.01	cellular and viral CCAAT box
		V$CLTR_CAAT.01	Mammalian C-type LTR CCAAT box
V$PDX1	Pancreatic and intestinal homeodomain transcr. factor	V$PDX1.01	Pdx1 (IDX1/IPF1) pancreatic and intestinal homeodomain TF
		V$ISL1.01	Pancreatic and intestinal lim-homeodomain factor
V$PERO	PEROxisome proliferator-activated receptor	V$PPARA.01	PPAR/RXR heterodimers
V$PIT1	GHF-1 pituitary specific pou domain transcription factor	V$PIT1.01	Pit1, GHF-1 pituitary specific pou domain transcription factor
V$RARF	Nuclear receptor for	V$RARA.01	Retinoic acid receptor,

Family	Family Information	Matrix Name	Information
	retenoic acid		member of nuclear receptors
		V$RTR.01	Retinoid receptor-related testis-associated receptor (GCNF/RTR)
V$RBIT	Regulator of B-Cell IgH transcription	V$BRIGHT.01	Bright, B cell regulator of IgH transcription
V$RBPF	RBPJ - kappa	V$RBPJK.01	Mammalian transcriptional repressor RBP-Jkappa/CBF1
V$REBV	Epstein-Barr virus transcription factor R	V$EBVR.01	Epstein-Barr virus transcription factor R
V$RORA	Estrogen receptor and rar-Rel. Orphan Receptor Alpha	V$RORA1.01	RAR-related orphan receptor alpha1
		V$RORA2.01	RAR-related orphan receptor alpha2
		V$ER.01	estrogen receptor
V$RREB	Ras-REsponsive element Binding protein	V$RREB1.01	Ras-responsive element binding protein 1
V$RXRF	RXR heterodimer binding sites	V$FXRE.01	Farnesoid X - activated receptor (RXR/FXR dimer)
		V$VDR_RXR.01	VDR/RXR Vitamin D receptor RXR heterodimer site
		V$VDR_RXR.02	VDR/RXR Vitamin D receptor RXR heterodimer site
		V$LXRE.01	Nuclear receptor involved in the regulation lipid homeostasis
V$SATB	Special AT-rich sequence binding protein	V$SATB1.01	Special AT-rich sequence-binding protein 1, predominantly expressed in thymocytes, binds to matrix

Family	Family Information	Matrix Name	Information
			attachment regions (MARs)
V$SEF1	SEF1 protein in mouse Retrovirus SL3-3	V$SEF1.01	SEF1 binding site
V$SF1F	Vertebrate steroidogenic factor	V$SF1.01	SF1 steroidogenic factor 1
V$SMAD	Vertebrate SMAD family of transcription factors	V$SMAD3.01	Smad3 transcription factor involved in TGF-beta signaling
		V$SMAD4.01	Smad4 transcription factor involved in TGF-beta signaling
		V$FAST1.01	FAST-1 SMAD interacting protein
V$SORY	SOx/sRY-sex/testis determinig and related HMG Box factors	V$SOX5.01	Sox-5
		V$SRY.01	sex-determining region Y gene product
		V$HMGIY.01	HMGI(Y) high-mobility-group protein I (Y), architectural transcription factor organizing the framework of a nuclear protein-DNA transcriptional complex
		V$SOX9.01	SOX (SRY-related HMG box)
V$SP1F	GC-Box factors_SP1/GC	V$SP1.01	stimulating protein 1 SP1, ubiquitous zinc finger transcription factor
		V$GC.01	GC box elements
V$SRFF	Serum Response element binding Factor	V$SRF.02	serum response factor
		V$SRF.03	serum responsive factor
		V$SRF.01	serum response factor

Family	Family Information	Matrix Name	Information
V$STAT	Signal Transducer and Activator of Transcript. factors	V$STAT.01	signal transducers and activators of transcription
		V$STAT5.01	STAT5: signal transducer and activator of transcription 5
		V$STAT6.01	STAT6: signal transducer and activator of transcription 6
		V$STAT1.01	signal transducer and activator of transcription 1
		V$STAT3.01	signal transducer and activator of transcription 3
V$T3RH	Viral homolog of thyroid hormon receptor alpha1 (AEV vErbA)	V$T3R.01	vErbA, viral homolog of thyroid hormone receptor alpha1
V$TBPF	Tata-Binding Protein Factor	V$TATA.02	Mammalian C-type LTR TATA box
		V$ATATA.01	Avian C-type LTR TATA box
		V$TATA.01	cellular and viral TATA box elements
		V$MTATA.01	Muscle TATA box
V$TCFF	TCF11 transcription Factor	V$TCF11.01	TCF11/KCR-F1/Nrf1 homodimers
V$TEAF	TEA/ATTS DNA binding domain factors	V$TEF1.01	TEF-1 related muscle factor
V$TTFF	Thyroid transcription factor-1	V$TTF1.01	Thyroid transcription factor-1 (TTF1) binding site
V$VBPF	chicken Vitellogenin gene Binding Protein factor	V$VBP.01	PAR-type chicken vitellogenin promoter-binding protein

Family	Family Information	Matrix Name	Information
V$VMYB	AMV-viral myb oncogene	V$VMYB.02	v-Myb
		V$VMYB.01	v-Myb
V$WHZF	Winged Helix and ZF5 binding sites	V$WHN.01	winged helix protein, involved in hair keratinization and thymus epithelium differentiation
V$XBBF	X-box binding Factors	V$RFX1.01	X-box binding protein RFX1
		V$RFX1.02	X-box binding protein RFX1
		V$MIF1.01	MIBP-1 / RFX1 complex
V$XSEC	Xenopus SEleno Cystein t-RNA activiating factor	V$STAF.02	Se-Cys tRNA gene transcription activating factor
		V$STAF.01	Se-Cys tRNA gene transcription activating factor
V$YY1F	activator/repressor binding to transcr. init. site	V$YY1.01	Yin and Yang 1
V$ZBPF	Zinc binding protein factor	V$ZBP89.01	Zinc finger transcription factor ZBP-89
V$ZFIA	ZincFinger with InterAction domain factors	V$ZID.01	zinc finger with interaction domain

B. Changes from Family Library Version 2.4 to Version 3.0

Matrix Family Library Version 3.0 (Nov 2002) contains 452 weight matrices in 216 families
(Vertebrates: 314 matrices in 128 families)

New weight matrices - Vertebrates

Family	Family Information	Matrix Name	Matrix Information
V$AP1F	AP1 and related factors	V$BACH1.01	BTB/POZ-bZIP transcription factor BACH1 forms heterodimers with the small Maf protein family
V$CIZF	CAS interating zinc finger protei	V$NMP4.01	NMP4 (nuclear matrix protein 4) / CIZ (Cas-interacting zinc finger protein)
V$CREB	Camp-Responsive Element Binding proteins	V$ATF6.02	Activating transcription factor 6, member of b-zip family, induced by ER stress
V$E4FF	Ubiquitous GLI - Krueppel like zinc finger involved in cell cycle regulation	V$E4F.01	GLI-Krueppel-related transcription factor, regulator of adenovirus E4 promoter
V$GFI1	Growth Factor Independence-transcriptional repressor	V$GfI1B.01	Growth factor independence 1 zinc finger protein Gfi-1B
V$GLIF	GLI zinc finger family	V$GLI1.01	Zinc finger transcription factor GLI1
V$HAML	Human Acute Myelogenous Leukemia factors	V$AML3.01	Runt-related transcription factor 2 / CBFA1 (core-binding factor, runt domain, alpha subunit 1)
V$HESF	Vertebrate homologues of enhancer of split complex	V$HES1.01	Drosophila hairy and enhancer of split homologue 1 (HES-1)
V$HIFF	Hypoxia inducible factor, bHLH / PAS protein family	V$HIF1.01	Hypoxia induced factor-1 (HIF-1)
		V$HIF1.02	Hypoxia inducible factor, bHLH / PAS protein family

Family	Family Information	Matrix Name	Matrix Information
V$HNF6	Onecut Homeodomain factor HNF6	V$HNF6.01	Liver enriched Cut - Homeodomain transcription factor HNF6 (ONECUT)
V$HOXF	Factors with moderate activity to homeo domain consensus sequence	V$CRX.01	Cone-rod homeobox-containing transcription factor / otx-like homeobox gene
		V$EN1.01	Homeobox protein engrailed (en-1)
		V$PTX1.01	Pituitary Homeobox 1 (Ptx1)
V$IRFF	Interferon Regulatory Factors	V$IRF3.01	Interferon regulatory factor 3 (IRF-3)
		V$IRF7.01	Interferon regulatory factor 7 (IRF-7)
V$MAZF	Myc associated zinc fingers	V$MAZ.01	Myc associated zinc finger protein (MAZ)
		V$MAZR.01	MYC-associated zinc finger protein related transcription factor
V$MEIS	Homeodomain factor aberrantly expressed in myeloid leukemia	V$MEIS1.01	Binding site for monomeric Meis1 homeodomain protein
V$MITF	Microphthalmia transcription factor	V$MIT.01	MIT (microphthalmia transcription factor) and TFE3
V$MOKF	Mouse Krueppel like factor	V$MOK2.02	Ribonucleoprotein associated zinc finger protein MOK-2 (human)
V$NEUR	NeuroD, Beta2, HLH domain	V$NEUROD1.01	DNA binding site for NEUROD1 (BETA-2 / E47 dimer)
V$NF1F	Nuclear Factor 1	V$NF1.02	Nuclear factor 1 (CTF1)
V$NKXH	NKX/DLX - Homeodomain sites	V$DLX1.01	DLX-1, -2, and -5 binding sites
		V$DLX3.01	Distal-less 3 homeodomain transcription facto
		V$HMX3.01	H6 homeodomain HMX3/Nkx5.1

Family	Family Information	Matrix Name	Matrix Information
			transcription factor
		V$MSX.01	Homeodomain proteins MSX-1 and MSX-2
		V$MSX2.01	Muscle segment homeo box 2, homologue of Drosophila (HOX 8)
V$NRLF	Neural retina leucine zipper	V$NRL.01	Neural retinal basic leucine zipper factor (bZIP)
V$PARF	PAR/bZIP family	V$DBP.01	Albumin D-box binding protein
V$PBXC	PBX1 - MEIS1 complexes	V$PBX1_MEIS1.01	Binding site for a Pbx1/Meis1 heterodimer
		V$PBX1_MEIS1.02	Binding site for a Pbx1/Meis1 heterodimer
		V$PBX1_MEIS1.03	Binding site for a Pbx1/Meis1 heterodimer
V$PLZF	C2H2 zinc finger protein PLZF	V$PLZF.01	Promyelocytic leukemia zink finger (TF with nine Krueppel-like zink fingers)
V$PXRF	Pregnane X receptor	V$PXRCAR.01	Halfsite of PXR (pregnane X receptor)/RXR resp. CAR (constitutive androstane receptor)/RXR heterodimer binding site
V$RORA	v-ERB and rar-related Orphan Receptor Alpha	V$NBRE.01	Monomers of the nur subfamily of nuclear receptors (nur77, nurr1, nor-1)
V$SF1F	Vertebrate steroidogenic factor	V$FTF.01	Alpha (1)-fetoprotein transcription factor (FTF), liver receptor homologue-1 (LHR-1)
V$SIXF	Sine oculis (SIX) homeodomain factors	V$SIX3.01	SIX3 / SIXdomain (SD) and Homeodomain (HD) transcription factor
V$TALE	TALE Homeodomain class recognizing TG motives	V$TGIF.01	TG-interacting factor belonging to TALE class of homeodomain factors

Family	Family Information	Matrix Name	Matrix Information
V$ZF5F	ZF5 POZ domain zinc finger	V$ZF5.01	Zinc finger / POZ domain transcription factor

Weight matrices renamed

- V$MEIS1.01 renamed to V$MEIS1_HOXA9.01

Weight matrices moved to other families

- V$BEL1.01 moved from V$AP1F to V$BEL1
- V$NF1.01 moved from V$MYOF to V$NF1
- V$ER.01 moved from V$RORA to V$EREF
- V$T3R.01 moved from V$T3RH to V$RORA
- V$CLTR_CAAT.01 moved from V$PCAT to V$RCAT
- V$FAST1.01 moved from V$SMAD to V$FAST

Weight matrices removed

- V$MUSCLE_INI.03

C. Changes from Family Library Version 3.0 to Version 3.1

Matrix Family Library Version 3.1 contains 456 weight matrices in 216 families (Vertebrates: 318 matrices in 128 families)

New weight matrices - Vertebrates

Family	Family Information	Matrix Name	Matrix Information
V$LEFF	LEF1/TCF	V$LEF1.02	TCF/LEF-1, involved in the Wnt signal transduction pathway
V$PAX2	PAX-2 binding sites	V$PAX2.01	Zebrafish PAX2 paired domain protein
V$PAX5	PAX-5/PAX-9 B-cell-specific activating protein	V$PAX5.03	PAX5 paired domain protein
V$PAX6	PAX-4/PAX-6 paired domain binding sites	V$PAX4_PD.01	PAX4 paired domain binding site
		V$PAX6.02	PAX6 paired domain and homeodomain are required for binding to this site
V$ZBPF	Zinc binding protein	V$ZF9.01	Core promoter-binding

	factor		protein (CPBP) with 3 Krueppel-type zinc fingers

Weight matrices modified

- V$AML1.01
- V$AML3.01

Weight matrices moved to other families

- V$ARNT.01 moved from V$EBOX to V$HIFF (ARNT is a synonym for HIF1 B)

Weight matrices removed

- V$SEF1.01
- V$OCT1.03

Version 3.1.1 (April 2003)

Matrices V$IRF3.01 and V$IRF7.01 corrected.

Version 3.1.2 (June 2003)

Matrix V$Gfi1B.01 corrected.

D. Changes from Family Library Version 3.1 to Version 3.3

Matrix Family Library Version 3.3 (August 2003) contains 485 weight matrices in 233 families
(Vertebrates: 326 matrices in 130 families)

New weight matrices - Vertebrates

Family	Family Information	Matrix Name	Matrix Information
V$EREF	Estrogen Response Elements	V$ER.02	Canonical palindromic estrogen response element (ERE)
V$SP1F	GC-Box factors_SP1/GC	V$BTEB3.01	Basic transcription element (BTE) binding protein, BTEB3, FKLF-2
V$CDEF	Cell cycle regulators: Cell cycle dependent element	V$CDE.01	Cell cycle-dependent element, CDF-1 binding site (CDE/CHR tandem elements regulate cell cycle dependent repression)
V$CHRF	Cell cycle regulators: Cell cycle homology element	V$CHR.01	Cell cycle gene homology region (CDE/CHR tandem elements regulate cell cycle dependent repression)
V$HIFF	Hypoxia inducible factor, bHLH / PAS protein family	V$CLOCK_BMAL1.01	Binding site of Clock/BMAL1 heterodimer, NPAS2/BMAL1 heterodimer
V$FKHD	Fork Head Domain factors	V$FKHRL1.01	Fkh-domain factor FKHRL1 (FOXO)
V$P53F	p53 tumor suppr.-neg. regulat. of the tumor suppr. Rb	V$P53.02	Tumor suppressor p53 (5' half site)
		V$P53.03	Tumor suppressor p53 (3' half site)

Weight matrices modified

- V$GFI1.01

E. Changes from Family Library Version 3.3 to Version 4.0

Matrix Family Library Version 4.0 (November 2003) contains 535 weight matrices in 253 families
(Vertebrates: 339 matrices in 136 families)

New weight matrices - Vertebrates

Family	Family Information	Matrix Name	Matrix Information
V$AARF	AARE binding factors	V$AARE.01	Amino acid response element, ATF4 binding site
V$AP1R	MAF and AP1 related factors	V$BACH2.01	Bach2 bound TRE
		V$NFE2L2.01	Nuclear factor (erythroid-derived 2)-like 2, NRF2
V$CDXF	Vertebrate caudal related homeodomain protein	V$CDX1.01	Intestine specific homeodomain factor CDX-1
V$DEAF	Homolog to deformed epidermal autoregulatory factor-1 from D. melanogaster	V$NUDR.01	NUDR (nuclear DEAF-1 related transcriptional regulator protein
V$ETSF	Human and murine ETS1 factors	V$ELF2.01	Ets - family member ELF-2 (NERF1a)
V$GABF	GA-boxes	V$GAGA.01	GAGA-Box
V$HNF1	Hepatic Nuclear Factor 1	V$HNF1.03	Hepatic nuclear factor 1
V$HOXF	Factors with moderate activity to homeo domain consensus sequence	V$GSC.01	Vertebrate bicoid-type homeodomain protein Goosecoid
V$LHXF	Lim homeodomain factors	V$LHX3.01	Homeodomain binding site in LIM/Homeodomain factor LHX3
V$NKXH	NKX/DLX - homeodomain sites	V$NKX32.01	Homeodomain protein NKX3.2 (BAPX1, NKX3B, Bagpipe homolog)
V$RBPF	RBPJ - kappa	V$RBPJK.02	Mammalian transcriptional repressor RBP-Jkappa/CBF1
V$RP58	RP58 (ZFP238) zinc finger protein	V$RP58.01	Zinc finger protein RP58 (ZNF238), associated preferentially with heterochromatin

Weight matrices modified

- V$GRE.01

- V$NFY.03

Weight matrices moved to other families

- V$BACH1.01 moved from V$AP1F to V$AP1R
- V$NFE2.01 moved from V$AP1F to V$AP1R
- V$TCF11MAFG.01 moved from V$AP1F to V$AP1R
- V$VMAF.01 moved from V$AP1F to V$AP1R

F. Changes from Family Library Version 4.0 to Version 4.1

Matrix Family Library Version 4.1 (February 2004) contains 564 weight matrices in 262 families
(Vertebrates: 356 matrices in 138 families)

New weight matrices - Vertebrates

Family	Family Information	Matrix Name	Matrix Information
V$BNCF	Basonuclein rDNA transcription factor (PolI)	V$BNC.01	Basonuclin, cooperates with USF1 in rDNA PolI transcription)
V$CMYB	C-myb, cellular transcriptional activator	V$CMYB.02	c-Myb, important in hematopoesis, cellular equivalent to avian myoblastosis virus oncogene v-myb
V$CP2F	CP2-erythrocyte Factor related to drosophila Elf1	V$CP2.02	LBP-1c (leader-binding protein-1c), LSF (late SV40 factor), CP2, SEF (SAA3 enhancer factor)
V$EKLF	Basic and erythroid Krueppel like factors	V$BKLF.01	Basic krueppel-like factor (KLF3)
V$HAND	bHLH transcription factor dimer of HAND2 and E12	V$HAND2_E12.01	Heterodimers of the bHLH transcription factors HAND2 (Thing2) and E12
V$HIFF	Hypoxia inducible factor, bHLH / PAS protein family	V$DEC1.01	Basic helix-loop-helix protein known as Dec1, Stra13 or Sharp2
V$HNF6	Onecut Homeodomain factor HNF6	V$OC2.01	CUT-homeodomain transcription factor Onecut-2
V$HOXF	Factors with moderate activity to homeo domain consensus sequence	V$OTX2.01	Homeodomain transcription factor Otx2 (homolog of Drosophila orthodenticle)

Family	Family Information	Matrix Name	Matrix Information
		V$GSH1.01	Homeobox transcription factor Gsh-1
V$IRFF	Interferon Regulatory Factors	V$IRF4.01	Interferon regulatory factor (IRF)-related protein (NF-EM5, PIP, LSIRF, ICSAT)
V$LHXF	Lim homeodomain factors	V$LMX1B.01	LIM-homeodomain transcription factor
V$MYT1	MYT1 C2HC zinc finger protein	V$MYT1L.01	Myelin transcription factor 1-like, neuronal C2HC zinc finger factor 1
V$NEUR	NeuroD, Beta2, HLH domain	V$NEUROG.01	Neurogenin 1 and 3 (ngn1/3) binding sites
V$VMYB	AMV-viral myb oncogene	V$VMYB.03	v-Myb, viral myb variant from transformed BM2 cells
		V$VMYB.04	v-Myb, AMV v-myb
		V$VMYB.05	v-Myb, variant of AMV v-myb
V$ZBPF	Zinc binding protein factor	V$ZNF202.01	Transcriptional repressor, binds to elements found predominantly in genes that participate in lipid metabolism

Weight matrices modified

- V$CMYB.01
- V$PTX1.01

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Example 6

Summary of Design for Particular Selectable Genes

TF binding sites and search parameters

Each TF binding site ("matrix") belongs to a matrix family that groups

10 functionally similar matrices together, eliminating redundant matches by MatInspector professional (the search program). Searches were limited to vertebrate TF binding sites. Searches were performed by matrix family, i.e., the results show only the best match from a family for each site. MatInspector

default parameters were used for the core and matrix similarity values (core similarity = 0.75, matrix similarity = optimized).

Table 18

Gene Designations

A. Synthetic hygromycin gene

Sequence	Description	Matrix Library
hyg	from pcDNA3.1/Hygro	Not applicable
hhyg	humanized ORF	Not applicable
hhyg-1	First removal of undesired sequence matches	Ver 3.1.2 Jun 2003
hhyg-2	Second removal of undesired sequence matches	Ver 3.1.2 Jun 2003
hhyg-3	Third removal of undesired sequence matches	Ver 3.1.2 Jun 2003
hHygro	Changes to ORF and add linker	Ver 3.3 Aug 2003
hhyg-4	Fourth removal of undesired sequence matches	Ver 3.3 Aug 2003

B. Synthetic neomycin gene

Sequence	Description	Matrix Library
neo	from pCI-neo or psiSTRIKE neo	Not applicable
hneo	humanized ORF	Not applicable
hneo-1	First removal of undesired sequence matches	Ver 3.1.2 Jun 2003
hneo-2	Second removal of undesired sequence matches	Ver 3.1.2 Jun 2003
hneo-3	Third removal of undesired sequence matches	Ver 3.1.2 Jun 2003
hneo-4	Changed 5′ and 3′ flanking regions/cloning sites	Ver 4.1 Feb 2004
hneo-5	Fourth removal of undesired sequence matches	Ver 4.1 Feb 2004

C. Synthetic puromycin gene

Sequence	Description	Matrix Library
puro	from psiSTRIKE puromycin	Not applicable
hpuro	humanized ORF	Not applicable
hpuro-1	First removal of undesired sequence matches	Ver 4.1 Feb 2004
hpuro-2	Second removal of undesired sequence matches	Ver 4.1 Feb 2004

Note: the above sequence names designate the ORF only (except for Hhygro which includes flanking sequences). Addition of "F" to the sequence name indicates the presence of up- and down-stream flanking sequences. Additional letters (e.g.,"B") indicate changes were made only to the flanking regions

Table 19

Sequences in Synthetic Hygromycin Genes

TFBS in hhyg

Before removal of TFBS from hhyg (94 matches)

Family/matrix	Further Information
V$PCAT/CAAT.01	cellular and viral CCAAT box
V$MINI/MUSCLE_INI.02	Muscle Initiator Sequence
V$MINI/MUSCLE_INI.01	Muscle Initiator Sequence
V$ETSF/PU1.01	Pu.1 (Pu120) Ets-like transcription factor identified in lymphoid B-cells
V$AHRR/AHRARNT.02	Aryl hydrocarbon / Arnt heterodimers, fixed core
V$EGRF/EGR3.01	early growth response gene 3 product
V$AP4R/AP4.01	Activator protein 4
V$EGRF/NGFIC.01	Nerve growth factor-induced protein C
V$MAZF/MAZ.01	Myc associated zinc finger protein (MAZ)
V$ZBPF/ZF9.01	Core promoter-binding protein (CPBP) with 3 Krueppel-type zinc fingers
V$CREB/ATF6.02	Activating transcription factor 6, member of b-zip family, induced by ER stress
V$EGRF/EGR3.01	early growth response gene 3 product
V$ZBPF/ZF9.01	Core promoter-binding protein (CPBP) with 3 Krueppel-type zinc fingers
V$HIFF/HIF1.02	Hypoxia inducible factor, bHLH / PAS

Family/matrix	Further Information
	protein family
V$E2FF/E2F.01	E2F, involved in cell cycle regulation, interacts with Rb p107 protein
V$AP4R/AP4.01	Activator protein 4
V$HEN1/HEN1.02	HEN1
V$MYOD/E47.01	MyoD/E47 and MyoD/E12 dimers
V$EGRF/EGR3.01	early growth response gene 3 product
V$MOKF/MOK2.02	Ribonucleoprotein associated zinc finger protein MOK-2 (human)
V$SP1F/GC.01	GC box elements
V$NRSF/NRSE.01	Neural-restrictive-silencer-element
V$RORA/RORA2.01	RAR-related orphan receptor alpha2
V$ZBPF/ZF9.01	Core promoter-binding protein (CPBP) with 3 Krueppel-type zinc fingers
V$ZF5F/ZF5.01	Zinc finger / POZ domain transcription factor
V$AHRR/AHRARNT.02	Aryl hydrocarbon / Arnt heterodimers, fixed core
V$AP1F/TCF11MAFG.01	TCF11/MafG heterodimers, binding to subclass of AP1 sites
V$EKLF/EKLF.01	Erythroid krueppel like factor (EKLF)
V$NRSF/NRSF.01	Neuron-restrictive silencer factor
V$NRSF/NRSE.01	Neural-restrictive-silencer-element
V$EBOX/MYCMAX.03	MYC-MAX binding sites
V$RXRF/FXRE.01	Farnesoid X - activated receptor (RXR/FXR dimer)
V$AHRR/AHRARNT.02	Aryl hydrocarbon / Arnt heterodimers, fixed core
V$WHZF/WHN.01	Winged helix protein, involved in hair keratinization and thymus epithelium differentiation
V$EGRF/EGR1.01	Egr-1/Krox-24/NGFI-A immediate-early gene product
V$SMAD/SMAD3.01	Smad3 transcription factor involved in TGF-beta signaling
V$MOKF/MOK2.01	Ribonucleoprotein associated zinc finger protein MOK-2 (mouse)
V$MYOD/MYOD.02	Myoblast determining factor

Family/matrix	Further Information
V$E4FF/E4F.01	GLI-Krueppel-related transcription factor, regulator of adenovirus E4 promoter
V$MOKF/MOK2.01	Ribonucleoprotein associated zinc finger protein MOK-2 (mouse)
V$EGRF/EGR2.01	Egr-2/Krox-20 early growth response gene product
V$EGRF/EGR3.01	early growth response gene 3 product
V$HIFF/HIF1.02	Hypoxia inducible factor, bHLH / PAS protein family
V$EBOX/USF.02	Upstream stimulating factor
V$HIFF/ARNT.01	AhR nuclear translocator homodimers
V$ZF5F/ZF5.01	Zinc finger / POZ domain transcription factor
V$EBOX/ATF6.01	Member of b-zip family, induced by ER damage/stress, binds to the ERSE in association with NF-Y
V$BEL1/BEL1.01	Bel-1 similar region (defined in Lentivirus LTRs)
V$NRSF/NRSE.01	Neural-restrictive-silencer-element
V$MYOD/MYOD.01	Myoblast determination gene product
V$NEUR/NEUROD1.01	DNA binding site for NEUROD1 (BETA-2 / E47 dimer)
V$AHRR/AHRARNT.01	Aryl hydrocarbon receptor / Arnt heterodimers
V$HIFF/ARNT.01	AhR nuclear translocator homodimers
V$VMYB/VMYB.02	v-Myb
V$MOKF/MOK2.01	Ribonucleoprotein associated zinc finger protein MOK-2 (mouse)
V$PAX5/PAX5.01	B-cell-specific activating protein
V$PBXC/PBX1_MEIS1.02	Binding site for a Pbx1/Meis1 heterodimer
V$MYOF/MYOGNF1.01	Myogenin / nuclear factor 1 or related factors
V$SRFF/SRF.03	Serum responsive factor
V$CP2F/CP2.01	CP2
V$OAZF/ROAZ.01	Rat C2H2 Zn finger protein involved in olfactory neuronal differentiation
V$AHRR/AHR.01	Aryl hydrocarbon / dioxin receptor
V$MINI/MUSCLE_INI.01	Muscle Initiator Sequence
V$PAX5/PAX5.02	B-cell-specific activating protein

Family/matrix	Further Information
V$ZBPF/ZF9.01	Core promoter-binding protein (CPBP) with 3 Krueppel-type zinc fingers
V$EBOX/ATF6.01	Member of b-zip family, induced by ER damage/stress, binds to the ERSE in association with NF-Y
V$EGRF/NGFIC.01	Nerve growth factor-induced protein C
V$ZF5F/ZF5.01	Zinc finger / POZ domain transcription factor
V$AP4R/AP4.02	Activator protein 4
V$XBBF/MIF1.01	MIBP-1 / RFX1 complex
V$EGRF/EGR3.01	early growth response gene 3 product
V$WHZF/WHN.01	Winged helix protein, involved in hair keratinization and thymus epithelium differentiation
V$PAX5/PAX5.01	B-cell-specific activating protein
V$WHZF/WHN.01	Winged helix protein, involved in hair keratinization and thymus epithelium differentiation
V$PAX5/PAX5.01	B-cell-specific activating protein
V$PAX5/PAX5.03	PAX5 paired domain protein
V$PAX5/PAX5.03	PAX5 paired domain protein
V$ZBPF/ZF9.01	Core promoter-binding protein (CPBP) with 3 Krueppel-type zinc fingers
V$CP2F/CP2.01	CP2
V$MINI/MUSCLE_INI.02	Muscle Initiator Sequence
V$AP2F/AP2.01	Activator protein 2
V$PAX5/PAX5.01	B-cell-specific activating protein
V$AHRR/AHRARNT.02	Aryl hydrocarbon / Arnt heterodimers, fixed core
V$MINI/MUSCLE_INI.02	Muscle Initiator Sequence
V$EGRF/EGR3.01	early growth response gene 3 product
V$SP1F/SP1.01	stimulating protein 1 SP1, ubiquitous zinc finger transcription factor
V$ZBPF/ZF9.01	Core promoter-binding protein (CPBP) with 3 Krueppel-type zinc fingers
V$EGRF/EGR1.01	Egr-1/Krox-24/NGFI-A immediate-early gene product
V$EGRF/WT1.01	Wilms Tumor Suppressor

Family/matrix	Further Information
V$SP1F/SP1.01	stimulating protein 1 SP1, ubiquitous zinc finger transcription factor
V$RCAT/CLTR_CAAT.01	Mammalian C-type LTR CCAAT box
V$ZBPF/ZF9.01	Core promoter-binding protein (CPBP) with 3 Krueppel-type zinc fingers
V$EGRF/WT1.01	Wilms Tumor Suppressor
V$EGRF/WT1.01	Wilms Tumor Suppressor
V$NF1F/NF1.01	Nuclear factor 1
V$PDX1/PDX1.01	Pdx1 (IDX1/IPF1) pancreatic and intestinal homeodomain TF

**matches are listed in order of occurrence in the corresponding sequence

TFBS in hhyg3

After removal of TFBS from hhyg2 (3 matches)

Family/matrix	Further Information
V$MINI/MUSCLE_INI.02	Muscle Initiator Sequence
V$PAX5/PAX5.02	B-cell-specific activating protein
V$VMYB/VMYB.02	v-Myb

**matches are listed in order of occurrence in the corresponding sequence

TFBS in hHygro

Before removal of TFBS from hHygro (5 matches, excluding linker)

Family/matrix	Further Information
V$MINI/MUSCLE_INI.02	Muscle Initiator Sequence
V$PAX5/PAX5.02	B-cell-specific activating protein
V$AREB/AREB6.04	AREB6 (Atp1a1 regulatory element binding factor 6)
V$VMYB/VMYB.02	v-Myb
V$CDEF/CDE.01	Cell cycle-dependent element, CDF-1 binding site (CDE/CHR tandem elements regulate cell cycle dependent repression)

**matches are listed in order of occurrence in the corresponding sequence

TFBS in hhyg4

After removal of TFBS from hHygro (4 matches)

Family/matrix**	Further Information
V$MINI/MUSCLE_INI.02	Muscle Initiator Sequence
V$PAX5/PAX5.02	B-cell-specific activating protein
V$AREB/AREB6.04	AREB6 (Atp1a1 regulatory element binding factor 6)
V$VMYB/VMYB.02	v-Myb

**matches are listed in order of occurrence in the corresponding sequence

Table 20

Sequences in Synthetic Neomycin Genes

TFBS in hneo

Before removal of TFBS from hneo (69 matches)

Family/matrix**	Further Information
V$PCAT/CAAT.01	cellular and viral CCAAT box
V$ZFIA/ZID.01	Zinc finger with interaction domain
V$AP1F/TCF11MAFG.01	TCF11/MafG heterodimers, binding to subclass of AP1 sites
V$MINI/MUSCLE_INI.01	Muscle Initiator Sequence
V$AHRR/AHRARNT.01	Aryl hydrocarbon receptor / Arnt heterodimers
V$HIFF/HIF1.02	Hypoxia inducible factor, bHLH / PAS protein family
V$SP1F/GC.01	GC box elements
V$MINI/MUSCLE_INI.02	Muscle Initiator Sequence
V$CP2F/CP2.01	CP2
V$WHZF/WHN.01	Winged helix protein, involved in hair keratinization and thymus epithelium differentiation
V$PAX5/PAX5.02	B-cell-specific activating protein
V$ZF5F/ZF5.01	Zinc finger / POZ domain transcription factor
V$ZBPF/ZF9.01	Core promoter-binding protein (CPBP) with 3 Krueppel-type zinc fingers
V$ZBPF/ZF9.01	Core promoter-binding protein (CPBP)

Family/matrix	Further Information
	with 3 Krueppel-type zinc fingers
V$HIFF/HIF1.02	Hypoxia inducible factor, bHLH / PAS protein family
V$AHRR/AHRARNT.01	Aryl hydrocarbon receptor / Arnt heterodimers
V$NRSF/NRSE.01	Neural-restrictive-silencer-element
V$HIFF/HIF1.02	Hypoxia inducible factor, bHLH / PAS protein family
V$CREB/ATF6.02	Activating transcription factor 6, member of b-zip family, induced by ER stress
V$RXRF/VDR_RXR.01	VDR/RXR Vitamin D receptor RXR heterodimer site
V$PCAT/CAAT.01	cellular and viral CCAAT box
V$NRSF/NRSE.01	Neural-restrictive-silencer-element
V$P53F/P53.01	Tumor suppressor p53
V$NEUR/NEUROD1.01	DNA binding site for NEUROD1 (BETA-2 / E47 dimer)
V$EBOX/USF.03	Upstream stimulating factor
V$MYOD/MYOD.02	Myoblast determining factor
V$NRSF/NRSE.01	Neural-restrictive-silencer-element
V$WHZF/WHN.01	Winged helix protein, involved in hair keratinization and thymus epithelium differentiation
V$EBOX/MYCMAX.03	MYC-MAX binding sites
V$HESF/HES1.01	Drosophila hairy and enhancer of split homologue 1 (HES-1)
V$NEUR/NEUROD1.01	DNA binding site for NEUROD1 (BETA-2 / E47 dimer)
V$MYOD/MYOD.02	Myoblast determining factor
V$REBV/EBVR.01	Epstein-Barr virus transcription factor R
V$PAX5/PAX5.02	B-cell-specific activating protein
V$ZF5F/ZF5.01	Zinc finger / POZ domain transcription factor
V$ZF5F/ZF5.01	Zinc finger / POZ domain transcription factor
V$EGRF/WT1.01	Wilms Tumor Suppressor
V$EGRF/WT1.01	Wilms Tumor Suppressor
V$ZBPF/ZF9.01	Core promoter-binding protein (CPBP)

Family/matrix	Further Information
	with 3 Krueppel-type zinc fingers
V$MINI/MUSCLE_INI.01	Muscle Initiator Sequence
V$NRSF/NRSF.01	Neuron-restrictive silencer factor
U$PflMI/PflMI	RE II-IP
V$NRSF/NRSE.01	Neural-restrictive-silencer-element
V$MOKF/MOK2.02	Ribonucleoprotein associated zinc finger protein MOK-2 (human)
V$AP2F/AP2.01	Activator protein 2
V$AP1F/AP1FJ.01	Activator protein 1
V$PAX5/PAX5.03	PAX5 paired domain protein
V$EGRF/EGR3.01	early growth response gene 3 product
V$WHZF/WHN.01	Winged helix protein, involved in hair keratinization and thymus epithelium differentiation
V$PAX6/PAX4_PD.01	PAX4 paired domain binding site
V$VMYB/VMYB.02	v-Myb
V$BEL1/BEL1.01	Bel-1 similar region (defined in Lentivirus LTRs)
V$MOKF/MOK2.01	Ribonucleoprotein associated zinc finger protein MOK-2 (mouse)
V$EGRF/EGR1.01	Egr-1/Krox-24/NGFI-A immediate-early gene product
V$EBOX/ATF6.01	Member of b-zip family, induced by ER damage/stress, binds to the ERSE in association with NF-Y
V$EGRF/EGR3.01	early growth response gene 3 product
V$NRSF/NRSE.01	Neural-restrictive-silencer-element
V$ETSF/ETS1.01	c-Ets-1 binding site
V$NRSF/NRSF.01	Neuron-restrictive silencer factor
V$SP1F/SP1 .01	stimulating protein 1 SP1, ubiquitous zinc finger transcription factor
V$ZBPF/ZBP89.01	Zinc finger transcription factor ZBP-89
V$PAX5/PAX5.03	PAX5 paired domain protein
V$GREF/ARE.01	Androgene receptor binding site
V$BCL6/BCL6.02	POZ/zinc finger protein, transcriptional repressor, translocations observed in diffuse large cell lymphoma
V$CLOX/CDP.01	cut-like homeodomain protein

**matches are listed in order of occurrence in the corresponding sequence

TFBS in hneo3

After removal of TFBS from hneo2 = before removal of TFBS from hneo3 (0 matches)

TFBS in hneo4

After removal of TFBS from hneo3 = before removal of TFBS from hneo4 (7 matches)

Family/matrix**	Further Information
V$PAX5/PAX9.01	Zebrafish PAX9 binding sites
V$AARF/AARE.01	Amino acid response element, ATF4 binding site
V$P53F/P53.02	Tumor suppressor p53 (5' half site)
V$AP1R/BACH2.01	Bach2 bound TRE
V$NEUR/NEUROG.01	Neurogenin 1 and 3 (ngn1/3) binding sites
V$CMYB/CMYB.01	c-Myb, important in hematopoesis, cellular equivalent to avian myoblastosis virus oncogene v-myb
V$HOXF/CRX.01	Cone-rod homeobox-containing transcription factor / otx-like homeobox gene

**matches are listed in order of occurrence in the corresponding sequence

TFBS in hneo5

After removal of TFBS from hneo4 (0 matches)

Table 21

Sequences in Synthetic Puromycin Genes

TFBS matches in hpuro

Before removal of TFBS from hpuro (68 matches)

Family/matrix**	Further Information
V$CDEF/CDE.01	Cell cycle-dependent element, CDF-1 binding site (CDE/CHR tandem elements regulate cell cycle dependent repression)
V$PAX3/PAX3.01	Pax-3 paired domain protein, expressed in embryogenesis, mutations correlate to Waardenburg

Family/matrix	Further Information
	Syndrome
V$CREB/ATF6.02	Activating transcription factor 6, member of b-zip family, induced by ER stress
V$EBOR/XBP1.01	X-box-binding protein 1
V$P53F/P53.03	Tumor suppressor p53 (3' half site)
V$HESF/HES1.01	Drosophila hairy and enhancer of split homologue 1 (HES-1)
V$MTF1/MTF-1.01	Metal transcription factor 1, MRE
V$EKLF/EKLF.01	Erythroid krueppel like factor (EKLF)
V$EGRF/EGR1.01	Egr-1/Krox-24/NGFI-A immediate-early gene product
V$EBOX/ATF6.01	Member of b-zip family, induced by ER damage/stress, binds to the ERSE in association with NF-Y
V$EBOX/ATF6.01	Member of b-zip family, induced by ER damage/stress, binds to the ERSE in association with NF-Y
V$CMYB/CMYB.01	c-Myb, important in hematopoesis, cellular equivalent to avian myoblastosis virus oncogene v-myb
V$AHRR/AHRARNT.01	Aryl hydrocarbon receptor / Arnt heterodimers
V$EBOX/MYCMAX.03	MYC-MAX binding sites
V$RORA/RORA2.01	RAR-related orphan receptor alpha2
V$EBOX/MYCMAX.03	MYC-MAX binding sites
V$HIFF/HIF1.02	Hypoxia inducible factor, bHLH / PAS protein family
V$EGRF/EGR3.01	early growth response gene 3 product
V$EGRF/WT1.01	Wilms Tumor Suppressor
V$HAML/AML3.01	Runt-related transcription factor 2 / CBFA1 (core-binding factor, runt domain, alpha subunit 1)
V$PAX5/PAX5.03	PAX5 paired domain protein
V$EBOX/ATF6.01	Member of b-zip family, induced by ER damage/stress, binds to the ERSE in association with NF-Y
V$HIFF/HIF1.02	Hypoxia inducible factor, bHLH /

Family/matrix	Further Information
	PAS protein family
V$ZBPF/ZBP89.01	Zinc finger transcription factor ZBP-89
V$OAZF/ROAZ.01	Rat C2H2 Zn finger protein involved in olfactory neuronal differentiation
V$GABF/GAGA.01	GAGA-Box
V$EBOX/MYCMAX.03	MYC-MAX binding sites
V$MYOD/MYF5.01	Myf5 myogenic bHLH protein
V$AP4R/TAL1BETAE47.01	Tal-1beta/E47 heterodimer
V$NEUR/NEUROG.01	Neurogenin 1 and 3 (ngn1/3) binding sites
V$HAND/HAND2_E12.01	Heterodimers of the bHLH transcription factors HAND2 (Thing2) and E12
V$MAZF/MAZR.01	MYC-associated zinc finger protein related transcription factor
V$ZBPF/ZNF202.01	Transcriptional repressor, binds to elements found predominantly in genes that participate in lipid metabolism
V$SP1F/SP1.01	Stimulating protein 1 SP1, ubiquitous zinc finger transcription factor
V$AP2F/AP2.01	Activator protein 2
V$RREB/RREB1.01	Ras-responsive element binding protein 1
V$XBBF/MIF1.01	MIBP-1 / RFX1 complex
V$CREB/TAXCREB.01	Tax/CREB complex
V$EGRF/EGR3.01	early growth response gene 3 product
V$MOKF/MOK2.01	Ribonucleoprotein associated zinc finger protein MOK-2 (mouse)
V$MOKF/MOK2.01	Ribonucleoprotein associated zinc finger protein MOK-2 (mouse)
V$PAX5/PAX5.01	B-cell-specific activating protein
V$NRSF/NRSE.01	Neural-restrictive-silencer-element
V$MINI/MUSCLE_INI.02	Muscle Initiator Sequence
V$EBOX/ATF6.01	Member of b-zip family, induced by ER damage/stress, binds to the ERSE in association with NF-Y
V$DEAF/NUDR.01	NUDR (nuclear DEAF-1 related

Family/matrix	Further Information
	transcriptional regulator protein)
V$AHRR/AHRARNT.01	Aryl hydrocarbon receptor / Arnt heterodimers
V$ZF5F/ZF5.01	Zinc finger / POZ domain transcription factor
V$EGRF/EGR1.01	Egr-1/Krox-24/NGFI-A immediate-early gene product
V$HIFF/HIF1.02	Hypoxia inducible factor, bHLH / PAS protein family
V$ETSF/ETS1.01	c-Ets-1 binding site
V$STAT/STAT1.01	Signal transducer and activator of transcription 1
V$BCL6/BCL6.01	POZ/zinc finger protein, transcriptional repressor, translocations observed in diffuse large cell lymphoma
V$ZF5F/ZF5.01	Zinc finger / POZ domain transcription factor
V$BCL6/BCL6.02	POZ/zinc finger protein, transcriptional repressor, translocations observed in diffuse large cell lymphoma
V$EGRF/EGR3.01	early growth response gene 3 product
V$CREB/ATF6.02	Activating transcription factor 6, member of b-zip family, induced by ER stress
V$HIFF/HIF1.02	Hypoxia inducible factor, bHLH / PAS protein family
V$EBOR/XBP1.01	X-box-binding protein 1
V$DEAF/NUDR.01	NUDR (nuclear DEAF-1 related transcriptional regulator protein)
V$RXRF/VDR_RXR.01	VDR/RXR Vitamin D receptor RXR heterodimer site
V$AP2F/AP2.01	Activator protein 2
V$REBV/EBVR.01	Epstein-Barr virus transcription factor R
V$ZBPF/ZF9.01	Core promoter-binding protein (CPBP) with 3 Krueppel-type zinc fingers
V$MYOD/LMO2COM.01	Complex of Lmo2 bound to Tal-1,

Family/matrix	Further Information
	E2A proteins, and GATA-1, half-site 1
V$AREB/AREB6.03	AREB6 (Atp1a1 regulatory element binding factor 6)
V$RXRF/FXRE.01	Farnesoid X - activated receptor (RXR/FXR dimer)
V$AHRR/AHR.01	Aryl hydrocarbon / dioxin receptor

**matches are listed in order of occurrence in the corresponding sequence

TFBS matches in hpuro1

After removal of TFBS from hpuro = before removal of TFBS from hpuro1 (4 matches)

Family/matrix	Further Information
V$NEUR/NEUROG.01	Neurogenin 1 and 3 (ngn1/3) binding sites
V$PAX5/PAX5.02	B-cell-specific activating protein
V$REBV/EBVR.01	Epstein-Barr virus transcription factor R
V$AHRR/AHR.01	Aryl hydrocarbon / dioxin receptor

**matches are listed in order of occurrence in the corresponding sequence

TFBS matches in hpuro2

After removal of TFBS from hpuro1 (2 matches)

Family/matrix	Further Information
V$NEUR/NEUROG.01	Neurogenin 1 and 3 (ngn1/3) binding sites
V$BCL6/BCL6.02	POZ/zinc finger protein, transcriptional repressor, translocations observed in diffuse large cell lymphoma

**matches are listed in order of occurrence in the corresponding sequence

Example 7

Summary of Design of Synthetic Firefly Luciferase Genes

TF binding sites and search parameters

The TF binding sites are from the TF binding site library ("Matrix Family Library") that is part of the GEMS Launcher package. Each TF binding site ("matrix") belongs to a matrix family that groups functionally similar matrices

together, eliminating redundant matches by MatInspector professional (the search program). Searches were limited to vertebrate TF binding sites. Searches were performed by matrix family, i.e. the results show only the best match from a family for each site. MatInspector default parameters were used for the core and matrix similarity values (core similarity = 0.75, matrix similarity = optimized).

Table 22

Luc Gene Designations

Synthetic luc gene (versions A and B)

Sequence*	Description	Matrix Library
Luc	wild-type gene	(not applicable)
luc+	improved gene from Promega's pGL3 vectors	(not applicable)
hluc+	Improved gene form Promega's pGL3(R2.1)-Basic	(not applicable)
	Codon optimization strategy A	
hluc+ver2A1	codon optimized luc+ (strategy A)	Ver 3.0 Nov 2002
hluc+ver2A2	First removal of undesired sequence matches	Ver 3.0 Nov 2002
hluc+ver2A3	Second removal of undesired sequence matches	Ver 3.0 Nov 2002
hluc+ver2A4	Third removal of undesired sequence matches	Ver 3.0 Nov 2002
hluc+ver2A5	Fourth removal of undesired sequence matches	Ver 3.0 Nov 2002
hluc+ver2A6	Fifth removal of undesired sequence matches	Ver 3.0 Nov 2002
hluc+ver2A7	Sixth removal of undesired sequence matches	Ver 3.1.1 Apr 2003
hluc+ver2A8	Removal of *BglI* (RE) site	Ver 3.1.1 Apr 2003
	Codon optimization strategy B	
hluc+ver2B1	codon optimized luc+ (strategy B)	Ver 3.0 Nov 2002
hluc+ver2B2	First removal of undesired sequence matches	Ver 3.0 Nov 2002
hluc+ver2B3	Second removal of undesired sequence matches	Ver 3.0 Nov 2002
hluc+ver2B4	Third removal of undesired sequence matches	Ver 3.0 Nov 2002
hluc+ver2B5	Fourth removal of undesired sequence matches	Ver 3.0 Nov 2002
hluc+ver2B6	Fifth removal of undesired sequence matches	Ver 3.0 Nov 2002
hluc+ver2B7	Sixth removal of undesired sequence matches	Ver 3.1.1 Apr 2003
hluc+ver2B8	Removal of *SmaI* (RE), Ptx1 (TF) sites	Ver 3.1.1 Apr 2003
hluc+ver2B9	Removal of additional CpG sequences	Ver 3.1.1 Apr 2003

Sequence*	Description	Matrix Library
hluc+ver2B10	Removal of *BglI* (RE) site	Ver 3.1.1 Apr 2003

* the sequence names designate open reading frames; RE = restriction enzyme recognition sequence

Table 23

Sequences in Synthetic Luc Genes (version A)

TFBS in hluc+ver2A1

Before removal of TFBS from hluc+ver2A1 (110 matches)

Family/matrix	Further Information
V$MINI/MUSCLE_INI.02	Muscle Initiator Sequence
V$WHZF/WHN.01	winged helix protein, involved in hair keratinization and thymus epithelium differentiation
V$GREF/PRE.01	Progesterone receptor binding site
V$MAZF/MAZR.01	MYC-associated zinc finger protein related transcription factor
V$SP1F/SP1.01	stimulating protein 1 SP1, ubiquitous zinc finger transcription factor
V$ZBPF/ZBP89.01	Zinc finger transcription factor ZBP-89
V$SF1F/SF1.01	SF1 steroidogenic factor 1
V$EGRF/NGFIC.01	Nerve growth factor-induced protein C
V$MINI/MUSCLE_INI.01	Muscle Initiator Sequence
V$EGRF/EGR2.01	Egr-2/Krox-20 early growth response gene product
V$ZF5F/ZF5.01	Zinc finger / POZ domain transcription factor
V$HESF/HES1.01	Drosophila hairy and enhancer of split homologue 1 (HES-1)
V$NRSF/NRSE.01	neural-restrictive-silencer-element
V$PAX5/PAX5.02	B-cell-specific activating protein
V$HAML/AML3.01	Runt-related transcription factor 2 / CBFA1 (core-binding factor, runt domain, alpha subunit 1)
V$GREF/PRE.01	Progesterone receptor binding site
V$P53F/P53.01	tumor suppressor p53
V$ZF5F/ZF5.01	Zinc finger / POZ domain transcription factor
V$EBOX/ATF6.01	Member of b-zip family, induced by ER damage/stress, binds to the ERSE in association with NF-Y
V$EGRF/EGR3.01	early growth response gene 3 product
V$NF1F/NF1.01	Nuclear factor 1

Family/matrix	Further Information
V$EGRF/EGR3.01	early growth response gene 3 product
V$REBV/EBVR.01	Epstein-Barr virus transcription factor R
V$MOKF/MOK2.01	Ribonucleoprotein associated zinc finger protein MOK-2 (mouse)
V$PBXC/PBX1_MEIS1.01	Binding site for a Pbx1/Meis1 heterodimer
V$XSEC/STAF.01	Se-Cys tRNA gene transcription activating factor
V$COMP/COMP1.01	COMP1, cooperates with myogenic proteins in multicomponent complex
V$MYOF/MYOGNF1.01	Myogenin / nuclear factor 1 or related factors
V$NEUR/NEUROD1.01	DNA binding site for NEUROD1 (BETA-2 / E47 dimer)
V$MYOD/MYOD.02	myoblast determining factor
V$AP2F/AP2.01	Activator protein 2
V$EVI1/EVI1.02	Ecotropic viral integration site 1 encoded factor
V$SMAD/SMAD4.01	Smad4 transcription factor involved in TGF-beta signaling
V$MYOD/MYF5.01	Myf5 myogenic bHLH protein
V$HESF/HES1.01	Drosophila hairy and enhancer of split homologue 1 (HES-1)
V$PAX5/PAX5.01	B-cell-specific activating protein
V$EBOX/ATF6.01	Member of b-zip family, induced by ER damage/stress, binds to the ERSE in association with NF-Y
V$SP1F/GC.01	GC box elements
V$MAZF/MAZR.01	MYC-associated zinc finger protein related transcription factor
V$RREB/RREB1.01	Ras-responsive element binding protein 1
V$AHRR/AHRARNT.01	Aryl hydrocarbon receptor / Arnt heterodimers
V$HIFF/HIF1.02	Hypoxia inducible factor, bHLH / PAS protein family
V$ZF5F/ZF5.01	Zinc finger / POZ domain transcription factor
V$EBOX/ATF6.01	Member of b-zip family, induced by ER damage/stress, binds to the ERSE in association with NF-Y
V$YY1F/YY1.01	Yin and Yang 1
V$ETSF/GABP.01	GABP: GA binding protein
V$MOKF/MOK2.01	Ribonucleoprotein associated zinc finger protein MOK-2 (mouse)
V$ETSF/ELK1.02	Elk-1
V$EBOX/MYCMAX.03	MYC-MAX binding sites
V$E4FF/E4F.01	GLI-Krueppel-related transcription factor, regulator of adenovirus E4 promoter

Family/matrix	Further Information
V$XBBF/RFX1.01	X-box binding protein RFX1
V$EVI1/EVI1.06	Ecotropic viral integration site 1 encoded factor
V$MOKF/MOK2.01	Ribonucleoprotein associated zinc finger protein MOK-2 (mouse)
V$NF1F/NF1.01	Nuclear factor 1
V$PBXC/PBX1_MEIS1.02	Binding site for a Pbx1/Meis1 heterodimer
V$ZF5F/ZF5.01	Zinc finger / POZ domain transcription factor
V$HESF/HES1.01	Drosophila hairy and enhancer of split homologue 1 (HES-1)
V$PAX5/PAX5.01	B-cell-specific activating protein
V$ETSF/GABP.01	GABP: GA binding protein
V$MYOD/MYOD.02	myoblast determining factor
V$XSEC/STAF.01	Se-Cys tRNA gene transcription activating factor
V$OAZF/ROAZ.01	Rat C2H2 Zn finger protein involved in olfactory neuronal differentiation
V$AP2F/AP2.01	Activator protein 2
V$PAX3/PAX3.01	Pax-3 paired domain protein, expressed in embryogenesis, mutations correlate to Waardenburg Syndrome
V$AP2F/AP2.01	Activator protein 2
V$MTF1/MTF-1.01	Metal transcription factor 1, MRE
V$SF1F/FTF.01	Alpha (1)-fetoprotein transcription factor (FTF), liver receptor homologue-1 (LHR-1)
V$SMAD/SMAD4.01	Smad4 transcription factor involved in TGF-beta signaling
V$NFKB/NFKAPPAB.01	NF-kappaB
V$EKLF/EKLF.01	Erythroid krueppel like factor (EKLF)
V$CREB/TAXCREB.01	Tax/CREB complex
V$E2FF/E2F.03	E2F, involved in cell cycle regulation, interacts with Rb p107 protein
V$CP2F/CP2.01	CP2
V$AHRR/AHRARNT.01	Aryl hydrocarbon receptor / Arnt heterodimers
V$EGRF/EGR2.01	Egr-2/Krox-20 early growth response gene product
V$ZF5F/ZF5.01	Zinc finger / POZ domain transcription factor
V$EBOR/XBP1.01	X-box-binding protein 1
V$FKHD/XFD3.01	Xenopus fork head domain factor 3
V$AP2F/AP2.01	Activator protein 2
V$EGRF/NGFIC.01	Nerve growth factor-induced protein C
V$PCAT/ACAAT.01	Avian C-type LTR CCAAT box

Family/matrix	Further Information
V$PBXC/PBX1_MEIS1.02	Binding site for a Pbx1/Meis1 heterodimer
V$AHRR/AHRARNT.02	Aryl hydrocarbon / Arnt heterodimers, fixed core
V$MOKF/MOK2.01	Ribonucleoprotein associated zinc finger protein MOK-2 (mouse)
V$GREF/GRE.01	Glucocorticoid receptor, C2C2 zinc finger protein binds glucocorticoid dependent to GREs
V$NEUR/NEUROD1.01	DNA binding site for NEUROD1 (BETA-2 / E47 dimer)
V$NRSF/NRSE.01	neural-restrictive-silencer-element
V$NRSF/NRSE.01	neural-restrictive-silencer-element
V$AHRR/AHRARNT.02	Aryl hydrocarbon / Arnt heterodimers, fixed core
V$EBOX/ATF6.01	Member of b-zip family, induced by ER damage/stress, binds to the ERSE in association with NF-Y
V$HIFF/HIF1.02	Hypoxia inducible factor, bHLH / PAS protein family
V$EGRF/EGR3.01	early growth response gene 3 product
V$EGRF/EGR3.01	early growth response gene 3 product
V$WHZF/WHN.01	winged helix protein, involved in hair keratinization and thymus epithelium differentiation
V$AP2F/AP2.01	Activator protein 2
V$HIFF/HIF1.02	Hypoxia inducible factor, bHLH / PAS protein family
V$NRSF/NRSE.01	neural-restrictive-silencer-element
V$ZFIA/ZID.01	zinc finger with interaction domain
V$SMAD/SMAD4.01	Smad4 transcription factor involved in TGF-beta signaling
V$AHRR/AHRARNT.02	Aryl hydrocarbon / Arnt heterodimers, fixed core
V$EBOX/MYCMAX.01	c-Myc/Max heterodimer
V$EBOX/USF.03	upstream stimulating factor
V$EGRF/EGR1.01	Egr-1/Krox-24/NGFI-A immediate-early gene product
V$MINI/MUSCLE_INI.01	Muscle Initiator Sequence
V$MOKF/MOK2.01	Ribonucleoprotein associated zinc finger protein MOK-2 (mouse)
V$NRSF/NRSE.01	neural-restrictive-silencer-element
V$NF1F/NF1.01	Nuclear factor 1
V$SF1F/SF1.01	SF1 steroidogenic factor 1

**matches are listed in order of occurrence in the corresponding sequence

TFBS in hluc+ver2A3

After removal of TFBS from hluc+ver2A2 = before removal of TFBS from hluc+ver2A3 (8 matches)

Family/matrix**	Further Information
V$EGRF/EGR2.01	Egr-2/Krox-20 early growth response gene product
V$HAML/AML3.01	Runt-related transcription factor 2 / CBFA1 (core-binding factor, runt domain, alpha subunit 1)
V$MYOF/MYOGNF1.01	Myogenin / nuclear factor 1 or related factors
V$NF1F/NF1.01	Nuclear factor 1
V$ETSF/GABP.01	GABP: GA binding protein
V$NFKB/NFKAPPAB.01	NF-kappaB
V$EKLF/EKLF.01	Erythroid krueppel like factor (EKLF)
V$FKHD/XFD3.01	Xenopus fork head domain factor 3

**matches are listed in order of occurrence in the corresponding sequence

TFBS in hluc+ver2A6

After removal of TFBS from hluc+ver2A5 (2 matches)

Family/matrix**	Further Information
V$HAML/AML3.01	Runt-related transcription factor 2 / CBFA1 (core-binding factor, runt domain, alpha subunit 1)
V$FKHD/XFD3.01	Xenopus fork head domain factor 3

**matches are listed in order of occurrence in the corresponding sequence

TFBS in hluc+ver2A6

Before removal of TFBS from hluc+ver2A6 (4 matches)

Family/matrix**	Further Information
V$PAX5/PAX5.03	PAX5 paired domain protein
V$LEFF/LEF1.02	TCF/LEF-1, involved in the Wnt signal transduction pathway
V$IRFF/IRF7.01	Interferon regulatory factor 7 (IRF-7)
V$FKHD/XFD3.01	Xenopus fork head domain factor 3

**matches are listed in order of occurrence in the corresponding sequence

TFBS in hluc+ver2A7

After removal of TFBS from hluc+ver2A6 = before removal of TFBS from hluc+ver2A7 (1 match)

Family/matrix	Further Information
V$FKHD/XFD3.01	Xenopus fork head domain factor 3

TFBS in hluc+ver2A8

After removal of TFBS from hluc+ver2A7 (1 match)

Family/matrix	Further Information
V$FKHD/XFD3.01	Xenopus fork head domain factor 3

Table 24

Sequences in Synthetic Luc Genes (version B)

TFBS in hluc+ver2B1

Before removal of TFBS from hluc+ver2B1 (187 matches)

Family/matrix	Further Information
V$HOXF/PTX1.01	Pituitary Homeobox 1 (Ptx1)
V$OCT1/OCT1.04	octamer-binding factor 1
V$OCTP/OCT1P.01	octamer-binding factor 1, POU-specific domain
V$NKXH/NKX25.02	homeo domain factor Nkx-2.5/Csx, tinman homolog low affinity sites
V$BARB/BARBIE.01	barbiturate-inducible element
V$TBPF/TATA.01	cellular and viral TATA box elements
V$GATA/GATA.01	GATA binding site (consensus)
V$AP4R/AP4.01	Activator protein 4
V$HEN1/HEN1.02	HEN1
V$SRFF/SRF.01	serum response factor
V$PARF/DBP.01	Albumin D-box binding protein
V$MOKF/MOK2.01	Ribonucleoprotein associated zinc finger protein MOK-2 (mouse)
V$EVI1/EVI1.04	Ecotropic viral integration site 1 encoded factor
V$GFI1/GfI1B.01	Growth factor independence 1 zinc finger protein Gfi-1B
V$RBPF/RBPJK.01	Mammalian transcriptional repressor RBP-Jkappa/CBF1
V$TBPF/TATA.02	Mammalian C-type LTR TATA box

Family/matrix	Further Information
V$AP4R/TAL1ALPHAE47.01	Tal-1alpha/E47 heterodimer
V$SRFF/SRF.01	serum response factor
V$OCTP/OCT1P.01	octamer-binding factor 1, POU-specific domain
V$BRNF/BRN2.01	POU factor Brn-2 (N-Oct 3)
V$CREB/E4BP4.01	E4BP4, bZIP domain, transcriptional repressor
V$VBPF/VBP.01	PAR-type chicken vitellogenin promoter-binding protein
V$EVI1/EVI1.04	Ecotropic viral integration site 1 encoded factor
V$CLOX/CDPCR3.01	cut-like homeodomain protein
V$GFI1/Gfi1B.01	Growth factor independence 1 zinc finger protein Gfi-1B
V$GATA/LMO2COM.02	complex of Lmo2 bound to Tal-1, E2A proteins, and GATA-1, half-site 2
V$SRFF/SRF.01	serum response factor
V$HOXT/MEIS1_HOXA9.01	Homeobox protein MEIS1 binding site
V$OCT1/OCT1.03	octamer-binding factor 1
V$GFI1/GFI1.01	Growth factor independence 1 zinc finger protein acts as transcriptional repressor
V$HNF6/HNF6.01	Liver enriched Cut - Homeodomain transcription factor HNF6 (ONECUT)
V$HAML/AML1.01	runt-factor AML-1
V$GREF/PRE.01	Progesterone receptor binding site
V$STAT/STAT5.01	STAT5: signal transducer and activator of transcription 5
V$TBPF/TATA.01	cellular and viral TATA box elements
V$CLOX/CDP.01	cut-like homeodomain protein
V$FKHD/HFH8.01	HNF-3/Fkh Homolog-8
V$FAST/FAST1.01	FAST-1 SMAD interacting protein
V$GFI1/Gfi1B.01	Growth factor independence 1 zinc finger protein Gfi-1B
V$CART/CART1.01	Cart-1 (cartilage homeoprotein 1)
V$HMTB/MTBF.01	muscle-specific Mt binding site
V$TBPF/TATA.01	cellular and viral TATA box elements
V$FKHD/XFD2.01	Xenopus fork head domain factor 2
V$BRNF/BRN2.01	POU factor Brn-2 (N-Oct 3)
V$MEF2/AMEF2.01	myocyte enhancer factor
V$BRNF/BRN2.01	POU factor Brn-2 (N-Oct 3)
V$BEL1/BEL1.01	Bel-1 similar region (defined in Lentivirus LTRs)
V$NOLF/OLF1.01	olfactory neuron-specific factor

Family/matrix	Further Information
V$OCT1/OCT1.06	octamer-binding factor 1
V$NFKB/NFKAPPAB.02	NF-kappaB
V$BCL6/BCL6.02	POZ/zinc finger protein, transcriptional repressor, translocations observed in diffuse large cell lymphoma
V$MOKF/MOK2.01	Ribonucleoprotein associated zinc finger protein MOK-2 (mouse)
V$HEAT/HSF1.01	heat shock factor 1
V$OCTP/OCT1P.01	octamer-binding factor 1, POU-specific domain
V$PIT1/PIT1.01	Pit1, GHF-1 pituitary specific pou domain transcription factor
V$HOXF/CRX.01	Cone-rod homeobox-containing transcription factor / otx-like homeobox gene
V$HNF6/HNF6.01	Liver enriched Cut - Homeodomain transcription factor HNF6 (ONECUT)
V$CLOX/CLOX.01	Clox
V$BCL6/BCL6.02	POZ/zinc finger protein, transcriptional repressor, translocations observed in diffuse large cell lymphoma
V$HOXF/PTX1.01	Pituitary Homeobox 1 (Ptx1)
V$GATA/GATA1.02	GATA-binding factor 1
V$FKHD/FREAC4.01	Fork head RElated ACtivator-4
V$E4FF/E4F.01	GLI-Krueppel-related transcription factor, regulator of adenovirus E4 promoter
V$PDX1/ISL1.01	Pancreatic and intestinal lim-homeodomain factor
V$CART/CART1.01	Cart-1 (cartilage homeoprotein 1)
V$GFI1/GFI1.01	Growth factor independence 1 zinc finger protein acts as transcriptional repressor
V$IRFF/IRF3.01	Interferon regulatory factor 3 (IRF-3)
V$BARB/BARBIE.01	barbiturate-inducible element
V$PBXF/PBX1.01	homeo domain factor Pbx-1
V$EVI1/EVI1.02	Ecotropic viral integration site 1 encoded factor
V$GATA/GATA2.01	GATA-binding factor 2
V$BRNF/BRN2.01	POU factor Brn-2 (N-Oct 3)
V$PARF/DBP.01	Albumin D-box binding protein
V$BRNF/BRN3.01	POU transcription factor Brn-3
V$ZBPF/ZBP89.01	Zinc finger transcription factor ZBP-89
V$CREB/TAXCREB.02	Tax/CREB complex
V$GREF/PRE.01	Progesterone receptor binding site
V$RBPF/RBPJK.01	Mammalian transcriptional repressor RBP-

Family/matrix	Further Information
	Jkappa/CBF1
V$GATA/GATA3.02	GATA-binding factor 3
V$STAT/STAT.01	signal transducers and activators of transcription
V$IKRS/IK2.01	Ikaros 2, potential regulator of lymphocyte differentiation
V$SRFF/SRF.01	serum response factor
V$SEF1/SEF1.01	SEF1 binding site
V$HAML/AML1.01	runt-factor AML-1
V$MOKF/MOK2.02	Ribonucleoprotein associated zinc finger protein MOK-2 (human)
V$FKHD/FREAC2.01	Fork head RElated ACtivator-2
V$HMTB/MTBF.01	muscle-specific Mt binding site
V$GFI1/GFI1.01	Growth factor independence 1 zinc finger protein acts as transcriptional repressor
V$ECAT/NFY.03	nuclear factor Y (Y-box binding factor)
V$HOXT/MEIS1_HOXA9.01	Homeobox protein MEIS1 binding site
V$PCAT/ACAAT.01	Avian C-type LTR CCAAT box
V$HNF6/HNF6.01	Liver enriched Cut - Homeodomain transcription factor HNF6 (ONECUT)
V$CLOX/CLOX.01	Clox
V$GATA/GATA3.02	GATA-binding factor 3
V$AREB/AREB6.04	AREB6 (Atp1a1 regulatory element binding factor 6)
V$GATA/GATA3.02	GATA-binding factor 3
V$FKHD/HNF3B.01	Hepatocyte Nuclear Factor 3beta
V$IRFF/IRF1.01	interferon regulatory factor 1
V$NKXH/NKX31.01	prostate-specific homeodomain protein NKX3.1
V$PBXF/PBX1.01	homeo domain factor Pbx-1
V$ECAT/NFY.03	nuclear factor Y (Y-box binding factor)
V$PBXC/PBX1_MEIS1.02	Binding site for a Pbx1/Meis1 heterodimer
V$CLOX/CDP.02	transcriptional repressor CDP
V$HOXT/MEIS1_HOXA9.01	Homeobox protein MEIS1 binding site
V$HOXF/HOXA9.01	Member of the vertebrate HOX - cluster of homeobox factors
V$GATA/GATA.01	GATA binding site (consensus)
V$NKXH/NKX31.01	prostate-specific homeodomain protein NKX3.1
V$GATA/GATA3.02	GATA-binding factor 3
V$HOXF/CRX.01	Cone-rod homeobox-containing transcription factor / otx-like homeobox gene
V$CART/CART1.01	Cart-1 (cartilage homeoprotein 1)

Family/matrix	Further Information
V$OCT1/OCT1.02	octamer-binding factor 1
V$MAZF/MAZR.01	MYC-associated zinc finger protein related transcription factor
V$ZBPF/ZBP89.01	Zinc finger transcription factor ZBP-89
V$GATA/GATA3.02	GATA-binding factor 3
V$HOXF/CRX.01	Cone-rod homeobox-containing transcription factor / otx-like homeobox gene
V$CLOX/CDPCR3.01	cut-like homeodomain protein
V$AP1F/VMAF.01	v-Maf
V$AP4R/TAL1ALPHAE47.01	Tal-1alpha/E47 heterodimer
V$PAX8/PAX8.01	PAX 2/5/8 binding site
V$BRAC/BRACH.01	Brachyury
V$GATA/GATA1.02	GATA-binding factor 1
V$RREB/RREB1.01	Ras-responsive element binding protein 1
V$MZF1/MZF1.01	MZF1
V$MOKF/MOK2.02	Ribonucleoprotein associated zinc finger protein MOK-2 (human)
V$HOXF/PTX1.01	Pituitary Homeobox 1 (Ptx1)
V$LTUP/TAACC.01	Lentiviral TATA upstream element
V$AP4R/TH1E47.01	Thing1/E47 heterodimer, TH1 bHLH member specific expression in a variety of embryonic tissues
V$XSEC/STAF.01	Se-Cys tRNA gene transcription activating factor
V$IKRS/IK3.01	Ikaros 3, potential regulator of lymphocyte differentiation
V$AP1F/AP1.01	AP1 binding site
V$MAZF/MAZ.01	Myc associated zinc finger protein (MAZ)
V$MZF1/MZF1.01	MZF1
V$CLOX/CDPCR3.01	cut-like homeodomain protein
V$P53F/P53.01	tumor suppressor p53
V$SMAD/SMAD3.01	Smad3 transcription factor involved in TGF-beta signaling
V$HMTB/MTBF.01	muscle-specific Mt binding site
V$OCT1/OCT1.03	octamer-binding factor 1
V$FKHD/XFD3.01	Xenopus fork head domain factor 3
V$PIT1/PIT1.01	Pit1, GHF-1 pituitary specific pou domain transcription factor
V$OCTP/OCT1P.01	octamer-binding factor 1, POU-specific domain
V$HOXF/HOX1-3.01	Hox-1.3, vertebrate homeobox protein

Family/matrix	Further Information
V$PBXF/PBX1.01	homeo domain factor Pbx-1
V$ECAT/NFY.03	nuclear factor Y (Y-box binding factor)
V$PBXC/PBX1_MEIS1.02	Binding site for a Pbx1/Meis1 heterodimer
V$CLOX/CDP.02	transcriptional repressor CDP
V$HOXT/MEIS1_HOXA9.01	Homeobox protein MEIS1 binding site
V$HOXF/HOXA9.01	Member of the vertebrate HOX - cluster of homeobox factors
V$GATA/GATA1.02	GATA-binding factor 1
V$PCAT/ACAAT.01	Avian C-type LTR CCAAT box
V$XSEC/STAF.01	Se-Cys tRNA gene transcription activating factor
V$OCTP/OCT1P.01	octamer-binding factor 1, POU-specific domain
V$CLOX/CDP.01	cut-like homeodomain protein
V$FAST/FAST1.01	FAST-1 SMAD interacting protein
V$ECAT/NFY.01	nuclear factor Y (Y-box binding factor)
V$MEF2/MMEF2.01	myocyte enhancer factor
V$TBPF/TATA.02	Mammalian C-type LTR TATA box
V$FAST/FAST1.01	FAST-1 SMAD interacting protein
V$LTUP/TAACC.01	Lentiviral TATA upstream element
V$MOKF/MOK2.01	Ribonucleoprotein associated zinc finger protein MOK-2 (mouse)
V$BRNF/BRN2.01	POU factor Brn-2 (N-Oct 3)
V$HOXF/CRX.01	Cone-rod homeobox-containing transcription factor / otx-like homeobox gene
V$NKXH/NKX31.01	prostate-specific homeodomain protein NKX3.1
V$HEN1/HEN1.01	HEN1
V$BEL1/BEL1.01	Bel-1 similar region (defined in Lentivirus LTRs)
V$HOXF/PTX1.01	Pituitary Homeobox 1 (Ptx1)
V$BRNF/BRN2.01	POU factor Brn-2 (N-Oct 3)
V$NFKB/NFKAPPAB.01	NF-kappaB
V$HAML/AML1.01	runt-factor AML-1
V$ZFIA/ZID.01	zinc finger with interaction domain
V$XSEC/STAF.02	Se-Cys tRNA gene transcription activating factor
V$IKRS/IK1.01	Ikaros 1, potential regulator of lymphocyte differentiation
V$FAST/FAST1.01	FAST-1 SMAD interacting protein
V$MOKF/MOK2.01	Ribonucleoprotein associated zinc finger protein MOK-2 (mouse)

Family/matrix**	Further Information
V$BEL1/BEL1.01	Bel-1 similar region (defined in Lentivirus LTRs)
V$EGRF/WT1.01	Wilms Tumor Suppressor
V$MAZF/MAZR.01	MYC-associated zinc finger protein related transcription factor
V$ZBPF/ZBP89.01	Zinc finger transcription factor ZBP-89
V$ZBPF/ZBP89.01	Zinc finger transcription factor ZBP-89
V$SP1F/GC.01	GC box elements
V$RREB/RREB1.01	Ras-responsive element binding protein 1
V$MOKF/MOK2.01	Ribonucleoprotein associated zinc finger protein MOK-2 (mouse)
V$MEIS/MEIS1.01	Binding site for monomeric Meis 1 homeodomain protein
V$BCL6/BCL6.02	POZ/zinc finger protein, transcriptional repressor, translocations observed in diffuse large cell lymphoma
V$GATA/GATA3.02	GATA-binding factor 3
V$HOXF/CRX.01	Cone-rod homeobox-containing transcription factor / otx-like homeobox gene
V$HOXF/CRX.01	Cone-rod homeobox-containing transcription factor / otx-like homeobox gene
V$MAZF/MAZR.01	MYC-associated zinc finger protein related transcription factor
V$MZF1/MZF1.01	MZF1
V$PDX1/PDX1.01	Pdx1 (IDX1/IPF1) pancreatic and intestinal homeodomain TF

**matches are listed in order of occurrence in the corresponding sequence

TFBS in hluc+ver2B3

After removal of TFBS from hluc+ver2B2 = before removal of TFBS from hluc+ver2B3 (35 matches)

Family/matrix**	Further Information
V$OCT1/OCT1.04	octamer-binding factor 1
V$BARB/BARBIE.01	barbiturate-inducible element
V$NFKB/NFKAPPAB.02	NF-kappaB
V$OCTP/OCT1P.01	octamer-binding factor 1, POU-specific domain
V$PIT1/PIT1.01	Pit1, GHF-1 pituitary specific pou domain transcription factor
V$HOXF/PTX1.01	Pituitary Homeobox 1 (Ptx1)
V$FKHD/FREAC4.01	Fork head RElated ACtivator-4

Family/matrix	Further Information
V$E4FF/E4F.01	GLI-Krueppel-related transcription factor, regulator of adenovirus E4 promoter
V$EVI1/EVI1.02	Ecotropic viral integration site 1 encoded factor
V$GATA/GATA2.01	GATA-binding factor 2
V$GREF/PRE.01	Progesterone receptor binding site
V$RBPF/RBPJK.01	Mammalian transcriptional repressor RBP-Jkappa/CBF1
V$STAT/STAT.01	signal transducers and activators of transcription
V$IKRS/IK2.01	Ikaros 2, potential regulator of lymphocyte differentiation
V$FKHD/FREAC2.01	Fork head RElated ACtivator-2
V$SRFF/SRF.01	serum response factor
V$GREF/PRE.01	Progesterone receptor binding site
V$CLOX/CDPCR3.01	cut-like homeodomain protein
V$AP4R/TAL1ALPHAE47.01	Tal-1alpha/E47 heterodimer
V$GATA/GATA1.02	GATA-binding factor 1
V$FKHD/XFD3.01	Xenopus fork head domain factor 3
V$PBXF/PBX1.01	homeo domain factor Pbx-1
V$ECAT/NFY.03	nuclear factor Y (Y-box binding factor)
V$PBXC/PBX1_MEIS1.02	Binding site for a Pbx1/Meis1 heterodimer
V$CLOX/CDP.02	transcriptional repressor CDP
V$HOXT/MEIS1_HOXA9.01	Homeobox protein MEIS1 binding site
V$HOXF/HOXA9.01	Member of the vertebrate HOX - cluster of homeobox factors
V$GATA/GATA1.02	GATA-binding factor 1
V$MINI/MUSCLE_INI.01	Muscle Initiator Sequence
V$CLOX/CDP.01	cut-like homeodomain protein
V$BRNF/BRN2.01	POU factor Brn-2 (N-Oct 3)
V$NFKB/NFKAPPAB.01	NF-kappaB
V$ZFIA/ZID.01	zinc finger with interaction domain
V$BCL6/BCL6.02	POZ/zinc finger protein, transcriptional repressor, translocations observed in diffuse large cell lymphoma
V$HOXF/CRX.01	Cone-rod homeobox-containing transcription factor / otx-like homeobox gene

**matches are listed in order of occurrence in the corresponding sequence

TFBS in hluc+ver2B6

After removal of TFBS from hluc+ver2B5 (2 matches)

Family/matrix**	Further Information
V$HOXF/PTX1.01	Pituitary Homeobox 1 (Ptx1)
V$FKHD/XFD3.01	Xenopus fork head domain factor 3

**matches are listed in order of occurrence in the corresponding sequence

TFBS in hluc+ver2B6

Before removal of TFBS from hluc+ver2B6 (6 matches)

Family/matrix**	Further Information
V$PAX6/PAX4_PD.01	PAX4 paired domain binding site
V$HOXF/PTX1.01	Pituitary Homeobox 1 (Ptx1)
V$FKHD/XFD3.01	Xenopus fork head domain factor 3
V$PAX6/PAX6.02	PAX6 paired domain and homeodomain are required for binding to this site
V$PAX5/PAX5.03	PAX5 paired domain protein
V$IRFF/IRF3.01	Interferon regulatory factor 3 (IRF-3)

**matches are listed in order of occurrence in the corresponding sequence

TFBS in hluc+ver2B7

After removal of TFBS from hluc+ver2B6 = before removal of TFBS from hluc+ver2B7 (2 matches)

Family/matrix**	Further Information
V$HOXF/PTX1.01	Pituitary Homeobox 1 (Ptx1)
V$FKHD/XFD3.01	Xenopus fork head domain factor 3

**matches are listed in order of occurrence in the corresponding sequence

TFBS in hluc+ver2B8

After removal of TFBS from hluc+ver2B7 = before removal of TFBS from hluc+ver2B8 (1 match)

Family/matrix**	Further Information
V$FKHD/XFD3.01	Xenopus fork head domain factor 3

TFBS in hluc+ver2B9

After removal of TFBS from hluc+ver2B8 = before removal of TFBS from hluc+ver2B9 (1 match)

Family/matrix	Further Information
V$FKHD/XFD3.01	Xenopus fork head domain factor 3

TFBS in hluc+ver2B10

After removal of TFBS from hluc+ver2B9 (1 match)

Family/matrix	Further Information
V$FKHD/XFD3.01	Xenopus fork head domain factor 3

Example 8

Summary of Design for pGL4 Sequences

Figure 2 depicts the design scheme for the pGL4 vector. A portion of the vector backbone in pGL3 which includes an *bla* gene and a sequence between *bla* and a multiple cloning region, but not a second open reading frame, was modified to yield pGL4. pGL4 includes an ampicillin resistance gene between a *Not*I and a *SpeI* site, the sequence of which was modified to remove regulatory sequences but not to optimize codons for mammalian expression (bla-1-bla-5), and a *Spe*I-*Nco*I fragment that includes a multiple cloning region and a translation trap. The translation trap includes about 60 nucleotides having at least two stop codons in each reading frame. The *Spe*I-*Nco*I fragment from a parent vector, pGL4-basics-5F2G-2, was modified to decrease undesired regulatory sequences (MCS-1 to MCS-4; SEQ ID Nos. 76-79). One of the resulting sequences, MCS-4, was combined with a modified ampicillin resistance gene, bla-5 (SEQ ID NO:84), to yield pGL4B-4NN (SEQ ID NO:95). pGL4B-4NN was further modified (pGL4-NN1-3; SEQ ID Nos. 96-98). To determine if additional polyA sequences in the *Spe*I-*Nco*I fragment further reduced expression from the vector backbone, various polyA sequences were inserted therein. For instance, pGL4NN-Blue Heron included a *c-mos* polyA sequence in the *Spe*I-*Nco*I fragment. However, removal of regulatory sequences in polyA sequences may alter the secondary structure and thus the function of those sequences.

In one vector, the *Spe*I-*Nco*I fragment from pGL3 (*Spe*I-*Nco*I start ver 2; SEQ ID NO:48) was modified to remove one transcription factor binding site and one restriction enzyme recognition site, and alter the multiple cloning region, yielding *Spe*I-*Nco*I ver2 (SEQ ID NO:49).

TF binding sites and search parameters

Each TF binding site ("matrix") belongs to a matrix family that groups functionally similar matrices together, eliminating redundant matches by MatInspector professional (the search program). Searches were limited to vertebrate TF binding sites. Searches were performed by matrix family, i.e., the results show only the best match from a family for each site. MatInspector default parameters were used for the core and matrix similarity values (core similarity = 0.75, matrix similarity = optimized), except for sequence MCS-1 (core similarity = 1.00, matrix similarity = optimized).

Table 25

Description of Designed Sequences

pGL4 sequences

Sequence	Description	Matrix Library
	SpeI-NcoI fragment with MCS, translation trap	
MCS-1	*Spe*I-*Nco*I from pGL4-basics-5F2G-2	Ver 2.2 Sep 2001
MCS-2	First removal of undesired sequence matches	Ver 2.2 Sep 2001
MCS-3	Second removal of undesired sequence matches	Ver 2.2 Sep 2001
MCS-4	Third removal of undesired sequence matches	Ver 2.3 Feb 2001
	NotI-SpeI fragment with bla gene	
Bla	Beta-lactamase gene from pGL3 vectors	
bla-1*	*Sac*II (RE) added, *Bsm*AI (RE) site removed (*)	Ver 2.2 Sep 2001
bla-2*	First removal of undesired sequence matches	Ver 2.3 Feb 2001
bla-3*	Second removal of undesired sequence matches	Ver 2.3 Feb 2001
bla-4*	Third removal of undesired sequence matches	Ver 2.3 Feb 2001

Sequence	Description	Matrix Library
bla-5*	Fourth removal of undesired sequence matches	Ver 2.3 Feb 2001
	NotI-NcoI fragment with bla, translation trap, MCS	
pGL4B-4NN	Combination of bla-5 and MCS-4 sections	Ver 2.4 May 2002
pGL4B-4NN1	First removal of undesired sequence matches	Ver 2.4 May 2002
pGL4B-4NN2	Second removal of undesired sequence matches	Ver 2.4 May 2002
pGL4B-4NN3	Third version after removal of CEBP (TF) site	Ver 2.4 May 2002
	SpeI-NcoI fragment with translation trap, polyA, MCS	
*Spe*I-*Nco*I-Ver2-start	Existing MCS replaced with new MCS	Ver 4.0 Nov 2003
*Spe*I-*Nco*I-Ver2	First removal of undesired sequence matches	Ver 4.0 Nov 2003

(*)Bla codon usage was not optimized for expression in mammalian cells. Low usage *E. coli* codons were avoided when changes were introduced to remove undesired sequence elements.

Table 26

Sequences in Synthetic *Spe*I-*Nco*I fragment of pGL4

TFBS in MCS-1

Before removal of TFBS from MCS-1 (14 matches)

Name of family matrix **	Further Information
V$PAX3/PAX3.01	Pax-3 paired domain protein, expressed in embryogenesis, mutations correlate to Waardenburg Syndrome
V$GATA/GATA.01	GATA binding site (consensus)
V$NKXH/NKX31.01	prostate-specific homeodomain protein

	NKX3.1
V$CREB/E4BP4.01	E4BP4, bZIP domain, transcriptional repressor
V$BRN2/BRN2.01	POU factor Brn-2 (N-Oct 3)
V$CREB/E4BP4.01	E4BP4, bZIP domain, transcriptional repressor
V$NKXH/NKX31.01	prostate-specific homeodomain protein NKX3.1
V$ZFIA/ZID.01	zinc finger with interaction domain
V$CP2F/CP2.01	CP2
V$BRAC/BRACH.01	Brachyury
V$PAX6/PAX6.01	Pax-6 paired domain protein
V$NKXH/NKX31.01	prostate-specific homeodomain protein NKX3.1
V$TEAF/TEF1.01	TEF-1 related muscle factor
V$ETSF/ELK1.02	Elk-1

**matches are listed in order of occurrence in the corresponding sequence

TFBS in MCS-2

After removal of TFBS from MCS-1 = before removal of TFBS from MCS-2 (12 matches)

Name of family/matrix **	Further Information

V$GATA/GATA.01	GATA binding site (consensus)
V$NKXH/NKX31.01	prostate-specific homeodomain protein NKX3.1
V$TBPF/ATATA.01	Avian C-type LTR TATA box
V$CART/CART1.01	Cart-1 (cartilage homeoprotein 1)
V$CREB/E4BP4.01	E4BP4, bZIP domain, transcriptional repressor
V$BRN2/BRN2.01	POU factor Brn-2 (N-Oct 3)
V$CREB/E4BP4.01	E4BP4, bZIP domain, transcriptional repressor
V$TBPF/ATATA.01	Avian C-type LTR TATA box
V$NKXH/NKX31.01	prostate-specific homeodomain protein NKX3.1
V$PAX6/PAX6.01	Pax-6 paired domain protein
V$PAX8/PAX8.01	PAX 2/5/8 binding site
V$PAX1/PAX1.01	Pax1 paired domain protein, expressed in the developing vertebral column of mouse embryos

**matches are listed in order of occurrence in the corresponding sequence

TFBS in MCS-3

After removal of TFBS from MCS-2 = before removal of TFBS from MCS-4 (0 matches)

TFBS in MCS-4

After removal of TFBS from MCS-3 (0 matches)

Table 27

Sequences in Synthetic *NotI-SpeI* Fragment of pGL4

TFBS in bla-1

Before removal of TFBS from bla-1 (94 matches)

Name of family/matrix	Further Information
V$GATA/GATA1.02	GATA-binding factor 1
V$HOXF/HOX1-3.01	Hox-1.3, vertebrate homeobox protein
V$TBPF/ATATA.01	Avian C-type LTR TATA box
V$ETSF/NRF2.01	nuclear respiratory factor 2
V$OCTP/OCT1P.01	octamer-binding factor 1, POU-specific domain
V$ETSF/ELK1.02	Elk-1
V$GKLF/GKLF.01	gut-enriched Krueppel-like factor
V$E2FF/E2F.02	E2F, involved in cell cycle regulation, interacts with Rb p107 protein
V$ETSF/NRF2.01	nuclear respiratory factor 2
V$AP1F/VMAF.01	v-Maf
V$XBBF/RFX1.01	X-box binding protein RFX1
V$AREB/AREB6.04	AREB6 (Atp1a1 regulatory element binding factor 6)
V$CMYB/CMYB.01	c-Myb, important in hematopoesis, cellular equivalent to avian myoblastosis virus oncogene v-myb
V$VMYB/VMYB.02	v-Myb
V$EBOX/NMYC.01	N-Myc
V$VBPF/VBP.01	PAR-type chicken vitellogenin promoter-binding protein
V$CMYB/CMYB.01	c-Myb, important in hematopoesis, cellular equivalent to avian

Name of family/matrix	Further Information
	myoblastosis virus oncogene v-myb
V$GATA/GATA3.02	GATA-binding factor 3
V$PAX8/PAX8.01	PAX 2/5/8 binding site
V$HNF4/HNF4.02	Hepatic nuclear factor 4
V$E2FF/E2F.01	E2F, involved in cell cycle regulation, interacts with Rb p107 protein
V$NFAT/NFAT.01	Nuclear factor of activated T-cells
V$ECAT/NFY.02	nuclear factor Y (Y-box binding factor)
V$TBPF/TATA.02	Mammalian C-type LTR TATA box
V$MYT1/MYT1.02	MyT1 zinc finger transcription factor involved in primary neurogenesis
V$GATA/GATA3.01	GATA-binding factor 3
V$CREB/CREB.02	cAMP-responsive element binding protein
V$WHZF/WHN.01	winged helix protein, involved in hair keratinization and thymus epithelium differentiation
V$IRFF/ISRE.01	interferon-stimulated response element
V$NRSF/NRSE.01	neural-restrictive-silencer-element
V$TCFF/TCF11.01	TCF11/KCR-F1/Nrf1 homodimers
V$STAT/STAT.01	signal transducers and activators of transcription
V$ECAT/NFY.03	nuclear factor Y (Y-box binding factor)
V$OCT1/OCT1.05	octamer-binding factor 1
V$OCTP/OCT1P.01	octamer-binding factor 1, POU-specific domain
V$NKXH/NKX25.02	homeo domain factor Nkx-2.5/Csx,

Name of family/matrix	Further Information
	tinman homolog low affinity sites
V$PIT1/PIT1.01	Pit1, GHF-1 pituitary specific pou domain transcription factor
V$CLOX/CDPCR3.01	cut-like homeodomain protein
V$GREF/ARE.01	Androgene receptor binding site
V$GATA/GATA1.04	GATA-binding factor 1
V$E2TF/E2.02	papilloma virus regulator E2
V$RPOA/POLYA.01	Mammalian C-type LTR Poly A signal
V$VMYB/VMYB.02	v-Myb
V$CEBP/CEBPB.01	CCAAT/enhancer binding protein beta
V$VBPF/VBP.01	PAR-type chicken vitellogenin promoter-binding protein
V$CREB/HLF.01	hepatic leukemia factor
V$SF1F/SF1.01	SF1 steroidogenic factor 1
V$XBBF/MIF1.01	MIBP-1 / RFX1 complex
V$IKRS/IK2.01	Ikaros 2, potential regulator of lymphocyte differentiation
V$MINI/MUSCLE_INI.02	Muscle Initiator Sequence
V$PCAT/CLTR_CAAT.01	Mammalian C-type LTR CCAAT box
V$PAX5/PAX5.01	B-cell-specific activating protein
V$RPAD/PADS.01	Mammalian C-type LTR Poly A downstream element
V$XBBF/RFX1.02	X-box binding protein RFX1
V$CEBP/CEBPB.01	CCAAT/enhancer binding protein beta
V$CREB/HLF.01	hepatic leukemia factor
V$HNF1/HNF1.01	hepatic nuclear factor 1

Name of family/matrix	Further Information
V$VMYB/VMYB.01	v-Myb
V$NKXH/NKX31.01	prostate-specific homeodomain protein NKX3.1
V$XBBF/RFX1.01	X-box binding protein RFX1
V$STAT/STAT.01	signal transducers and activators of transcription
V$HNF1/HNF1.01	hepatic nuclear factor 1
V$HMYO/S8.01	S8
V$SORY/SOX5.01	Sox-5
V$RBIT/BRIGHT.01	Bright, B cell regulator of IgH transcription
V$NKXH/NKX25.02	homeo domain factor Nkx-2.5/Csx, tinman homolog low affinity sites
V$GATA/GATA1.02	GATA-binding factor 1
V$BARB/BARBIE.01	barbiturate-inducible element
V$MTF1/MTF-1.01	Metal transcription factor 1, MRE
V$NFKB/CREL.01	c-Rel
V$ETSF/ELK1.02	Elk-1
V$CLOX/CDP.01	cut-like homeodomain protein
V$RPOA/LPOLYA.01	Lentiviral Poly A signal
V$GATA/GATA1.03	GATA-binding factor 1
V$ZFIA/ZID.01	zinc finger with interaction domain
V$WHZF/WHN.01	winged helix protein, involved in hair keratinization and thymus epithelium differentiation
V$PAX1/PAX1.01	Pax1 paired domain protein, expressed in the developing vertebral column of

Name of family/matrix**	Further Information
	mouse embryos
V$GATA/LMO2COM.02	complex of Lmo2 bound to Tal-1, E2A proteins, and GATA-1, half-site 2
V$NRSF/NRSF.01	neuron-restrictive silencer factor
V$AP4R/TAL1BETAE47.01	Tal-1beta/E47 heterodimer
V$GATA/LMO2COM.02	complex of Lmo2 bound to Tal-1, E2A proteins, and GATA-1, half-site 2
V$GATA/GATA1.02	GATA-binding factor 1
V$XBBF/RFX1.01	X-box binding protein RFX1
V$AHRR/AHRARNT.02	aryl hydrocarbon / Arnt heterodimers, fixed core
V$PAX5/PAX9.01	zebrafish PAX9 binding sites
V$CLOX/CDP.02	transcriptional repressor CDP
V$GATA/GATA1.01	GATA-binding factor 1
V$AP1F/TCF11MAFG.01	TCF11/MafG heterodimers, binding to subclass of AP1 sites
V$BRN2/BRN2.01	POU factor Brn-2 (N-Oct 3)
V$NKXH/NKX25.02	homeo domain factor Nkx-2.5/Csx, tinman homolog low affinity sites
V$ECAT/NFY.02	nuclear factor Y (Y-box binding factor)
V$FKHD/FREAC4.01	Fork head RElated ACtivator-4
V$NFAT/NFAT.01	Nuclear factor of activated T-cells
V$IRFF/IRF1.01	interferon regulatory factor 1
V$E2FF/E2F.02	E2F, involved in cell cycle regulation, interacts with Rb p107 protein

**matches are listed in order of occurrence in the corresponding sequence

TFBS in bla-2

After removal of TFBS from bla-1 = before removal of TFBS from bla-2 = (51 matches)

Name of family/matrix	Further Information
V$GATA/GATA1.02	GATA-binding factor 1
V$ETSF/NRF2.01	nuclear respiratory factor 2
V$OCTP/OCT1P.01	octamer-binding factor 1, POU-specific domain
V$ETSF/ELK1.02	Elk-1
V$EBOX/NMYC.01	N-Myc
V$GATA/GATA3.02	GATA-binding factor 3
V$PAX8/PAX8.01	PAX 2/5/8 binding site
V$HNF4/HNF4.02	Hepatic nuclear factor 4
V$E2FF/E2F.01	E2F, involved in cell cycle regulation, interacts with Rb p107 protein
V$NFAT/NFAT.01	Nuclear factor of activated T-cells
V$ECAT/NFY.02	nuclear factor Y (Y-box binding factor)
V$TBPF/TATA.02	Mammalian C-type LTR TATA box
V$MYT1/MYT1.02	MyT1 zinc finger transcription factor involved in primary neurogenesis
V$GATA/GATA3.01	GATA-binding factor 3
V$CREB/CREB.02	cAMP-responsive element binding protein
V$WHZF/WHN.01	winged helix protein, involved in hair keratinization and thymus epithelium differentiation
V$NRSF/NRSE.01	neural-restrictive-silencer-element
V$OCT1/OCT1.05	octamer-binding factor 1
V$CLOX/CDPCR3.01	cut-like homeodomain protein

Name of family/matrix **	Further Information
V$GREF/ARE.01	Androgene receptor binding site
V$GATA/GATA1.04	GATA-binding factor 1
V$CEBP/CEBPB.01	CCAAT/enhancer binding protein beta
V$CREB/HLF.01	hepatic leukemia factor
V$VBPF/VBP.01	PAR-type chicken vitellogenin promoter-binding protein
V$XBBF/MIF1.01	MIBP-1 / RFX1 complex
V$IKRS/IK2.01	Ikaros 2, potential regulator of lymphocyte differentiation
V$PAX5/PAX5.01	B-cell-specific activating protein
V$XBBF/RFX1.02	X-box binding protein RFX1
V$CEBP/CEBPB.01	CCAAT/enhancer binding protein beta
V$CREB/HLF.01	hepatic leukemia factor
V$XBBF/RFX1.02	X-box binding protein RFX1
V$GATA/GATA1.02	GATA-binding factor 1
V$BARB/BARBIE.01	barbiturate-inducible element
V$MTF1/MTF-1.01	Metal transcription factor 1, MRE
V$NFKB/CREL.01	c-Rel
V$ETSF/ELK1.02	Elk-1
V$TBPF/TATA.01	cellular and viral TATA box elements
V$MEIS/MEIS1.01	Homeobox protein MEIS1 binding site
V$HOXF/HOXA9.01	Member of the vertebrate HOX - cluster of homeobox factors
V$GATA/GATA1.03	GATA-binding factor 1
V$MEIS/MEIS1.01	Homeobox protein MEIS1 binding site
V$NOLF/OLF1.01	olfactory neuron-specific factor

Name of family/matrix **	Further Information
V$AP4R/TAL1BETAE47.01	Tal-1beta/E47 heterodimer
V$GATA/GATA1.02	GATA-binding factor 1
V$XBBF/RFX1.01	X-box binding protein RFX1
V$AHRR/AHRARNT.02	aryl hydrocarbon / Arnt heterodimers, fixed core
V$PAX5/PAX9.01	zebrafish PAX9 binding sites
V$CLOX/CDP.02	transcriptional repressor CDP
V$GATA/GATA1.01	GATA-binding factor 1
V$IRFF/IRF1.01	interferon regulatory factor 1
V$E2FF/E2F.02	E2F, involved in cell cycle regulation, interacts with Rb p107 protein

**matches are listed in order of occurrence in the corresponding sequence

TFBS in bla-3

After removal of TFBS from bla-2 = before removal of TFBS from bla-3 = (16 matches)

Name of family/matrix **	Further Information
V$ETSF/NRF2.01	nuclear respiratory factor 2
V$E2FF/E2F.02	E2F, involved in cell cycle regulation, interacts with Rb p107 protein
V$NFAT/NFAT.01	Nuclear factor of activated T-cells
V$TBPF/TATA.02	Mammalian C-type LTR TATA box
V$MYT1/MYT1.02	MyT1 zinc finger transcription factor involved in primary neurogenesis

Name of family/matrix**	Further Information
V$WHZF/WHN.01	winged helix protein, involved in hair keratinization and thymus epithelium differentiation
V$SORY/SOX5.01	Sox-5
V$CEBP/CEBPB.01	CCAAT/enhancer binding protein beta
V$CREB/HLF.01	hepatic leukemia factor
V$VBPF/VBP.01	PAR-type chicken vitellogenin promoter-binding protein
V$PAX5/PAX5.01	B-cell-specific activating protein
V$XBBF/RFX1.02	X-box binding protein RFX1
V$CREB/HLF.01	hepatic leukemia factor
V$GATA/GATA1.03	GATA-binding factor 1
V$MEIS/MEIS1.01	Homeobox protein MEIS1 binding site
V$NOLF/OLF1.01	olfactory neuron-specific factor

**matches are listed in order of occurrence in the corresponding sequence

TFBS in bla-4

After removal of TFBS from bla-3 = before removal of TFBS from bla-4 = (14 matches)

Name of family/matrix**	Further Information

Name of family/matrix**	Further Information
V$ETSF/NRF2.01	nuclear respiratory factor 2
V$NFAT/NFAT.01	Nuclear factor of activated T-cells
V$WHZF/WHN.01	winged helix protein, involved in hair keratinization and thymus epithelium differentiation
V$GATA/GATA3.01	GATA-binding factor 3
V$CEBP/CEBPB.01	CCAAT/enhancer binding protein beta
V$EBOX/USF.02	upstream stimulating factor
V$PAX5/PAX5.01	B-cell-specific activating protein
V$XBBF/RFX1.02	X-box binding protein RFX1
V$GATA/GATA1.03	GATA-binding factor 1
V$MEIS/MEIS1.01	Homeobox protein MEIS1 binding site
V$ZFIA/ZID.01	zinc finger with interaction domain
V$WHZF/WHN.01	winged helix protein, involved in hair keratinization and thymus epithelium differentiation
V$PAX1/PAX1.01	Pax1 paired domain protein, expressed in the developing vertebral column of mouse embryos
V$GATA/LMO2COM.02	complex of Lmo2 bound to Tal-1, E2A

Name of family/matrix**	Further Information
	proteins, and GATA-1, half-site 2

**matches are listed in order of occurrence in the corresponding sequence

TFBS in bla-5

After removal of TFBS from bla-4 (5 matches)

Name of family/matrix**	Further Information
V$ETSF/NRF2.01	nuclear respiratory factor 2
V$WHZF/WHN.01	winged helix protein, involved in hair keratinization and thymus epithelium differentiation
V$GATA/GATA3.01	GATA-binding factor 3
V$CEBP/CEBPB.01	CCAAT/enhancer binding protein beta
V$EBOX/USF.02	upstream stimulating factor

**matches are listed in order of occurrence in the corresponding sequence

Table 28

Sequences in Synthetic *Not*I-*Nco*I Fragment of pGL4

TFBS in pGL4B-4NN

Before removal of TFBS from pGL4B-4NN = (11 matches)

Name of family/matrix**	Further Information
V$SMAD/FAST1.01	FAST-1 SMAD interacting protein
V$SMAD/FAST1.01	FAST-1 SMAD interacting protein

V$ETSF/FLI.01	ETS family member FLI
V$RBPF/RBPJK.01	Mammalian transcriptional repressor RBP-Jkappa/CBF1
V$ETSF/FLI.01	ETS family member FLI
V$EBOX/USF.02	upstream stimulating factor
V$CEBP/CEBPB.01	CCAAT/enhancer binding protein beta
V$GATA/GATA3.01	GATA-binding factor 3
V$WHZF/WHN.01	winged helix protein, involved in hair keratinization and thymus epithelium differentiation
V$ETSF/NRF2.01	nuclear respiratory factor 2
V$TBPF/ATATA.01	Avian C-type LTR TATA box

**matches are listed in order of occurrence in the corresponding sequence

TFBS in pGL4B-4NN1

After removal of TFBS from pGL4B-4NN = before removal of TFBS from pGL4B-4NN1 (7 matches)

Name of family/matrix**	Further Information
V$ETSF/NRF2.01	nuclear respiratory factor 2
V$WHZF/WHN.01	winged helix protein, involved in hair keratinization and thymus epithelium differentiation
V$CEBP/CEBPB.01	CCAAT/enhancer binding protein beta

V$EBOX/USF.02	upstream stimulating factor
V$ETSF/FLI.01	ETS family member FLI
V$SMAD/FAST1.01	FAST-1 SMAD interacting protein
V$SMAD/FAST1.01	FAST-1 SMAD interacting protein

**matches are listed in order of occurrence in the corresponding sequence

TFBS in pGL4B-4NN2

After removal of TFBS from pGL4B-4NN1 = before removal of TFBS from pGL4B-4NN2 (4 matches)

Name of family/matrix **	Further Information
V$ETSF/NRF2.01	nuclear respiratory factor 2
V$WHZF/WHN.01	winged helix protein, involved in hair keratinization and thymus epithelium differentiation
V$CEBP/CEBPB.01	CCAAT/enhancer binding protein beta
V$EBOX/USF.02	upstream stimulating factor

**matches are listed in order of occurrence in the corresponding sequence

TFBS in pGL4B-4NN3

After removal of TFBS from pGL4B-4NN2 (3 matches)

Name of family/matrix **	Further Information
V$EBOX/USF.	upstream stimulating factor

02	
V$WHZF/WHN.01	winged helix protein, involved in hair keratinization and thymus epithelium differentiation
V$ETSF/NRF2.01	nuclear respiratory factor 2

**matches are listed in order of occurrence in the corresponding sequence

Table 29

Sequences in Synthetic *Spe*I-*Nco*I section of pGL4

TFBS in *Spe*I-*Nco*I-Ver2-start

Before removal of TFBS from *Spe*I-*Nco*I-Ver2-start (34 matches)

Family/matrix	Further Information
V$PAX8/PAX8.01	PAX 2/5/8 binding site
V$GATA/GATA1.02	GATA-binding factor 1
V$CREB/E4BP4.01	E4BP4, bZIP domain, transcriptional repressor
V$NKXH/NKX31.01	Prostate-specific homeodomain protein NKX3.1
V$TBPF/ATATA.01	Avian C-type LTR TATA box
V$CREB/E4BP4.01	E4BP4, bZIP domain, transcriptional repressor
V$NKXH/NKX31.01	Prostate-specific homeodomain protein NKX3.1
V$CART/CART1.01	Cart-1 (cartilage homeoprotein 1)
V$NKXH/NKX25.02	Homeo domain factor Nkx-2.5/Csx, tinman homolog low affinity sites
V$ETSF/ELK1.01	Elk-1

Family/matrix	Further Information
V$CDXF/CDX2.01	Cdx-2 mammalian caudal related intestinal transcr. factor
V$BRNF/BRN3.01	POU transcription factor Brn-3
V$TBPF/TATA.02	Mammalian C-type LTR TATA box
V$FKHD/FREAC3.01	Fork head related activator-3 (FOXC1)
V$OCT1/OCT1.02	Octamer-binding factor 1
V$CART/CART1.01	Cart-1 (cartilage homeoprotein 1)
V$PDX1/PDX1.01	Pdx1 (IDX1/IPF1) pancreatic and intestinal homeodomain TF
V$PARF/DBP.01	Albumin D-box binding protein
V$GATA/GATA3.02	GATA-binding factor 3
V$VBPF/VBP.01	PAR-type chicken vitellogenin promoter-binding protein
V$AP4R/TAL1ALPHAE47.01	Tal-1alpha/E47 heterodimer
V$RP58/RP58.01	Zinc finger protein RP58 (ZNF238), associated preferentially with heterochromatin
V$COMP/COMP1.01	COMP1, cooperates with myogenic proteins in multicomponent complex
V$CLOX/CLOX.01	Clox
V$TBPF/ATATA.01	Avian C-type LTR TATA box
V$PBXC/PBX1_MEIS1.02	Binding site for a Pbx1/Meis1 heterodimer
V$PBXF/PBX1.01	Homeo domain factor Pbx-1
V$IRFF/IRF1.01	Interferon regulatory factor 1
V$TEAF/TEF1.01	TEF-1 related muscle factor

Family/matrix	Further Information
V$EBOX/ATF6.01	Member of b-zip family, induced by ER damage/stress, binds to the ERSE in association with NF-Y
V$NKXH/NKX32.01	Homeodomain protein NKX3.2 (BAPX1, NKX3B, Bagpipe homolog)
V$E2TF/E2.02	Papilloma virus regulator E2
V$EVI1/EVI1.05	Ecotropic viral integration site 1 encoded factor
V$GATA/GATA3.02	GATA-binding factor 3

**matches are listed in order of occurrence in the corresponding sequence

TFBS in *Spe*I-*Nco*I-Ver2

After removal of TFBS from *Spe*I-*Nco*I-Ver2-start (28 matches)

Family/matrix	Further Information
V$PAX8/PAX8.01	PAX 2/5/8 binding site
V$GATA/GATA1.02	GATA-binding factor 1
V$CREB/E4BP4.01	E4BP4, bZIP domain, transcriptional repressor
V$NKXH/NKX31.01	Prostate-specific homeodomain protein NKX3.1
V$TBPF/ATATA.01	Avian C-type LTR TATA box
V$CREB/E4BP4.01	E4BP4, bZIP domain, transcriptional repressor
V$NKXH/NKX31.01	Prostate-specific homeodomain protein NKX3.1
V$CART/CART1.01	Cart-1 (cartilage homeoprotein 1)

Family/matrix	Further Information
V$NKXH/NKX25.02	Homeo domain factor Nkx-2.5/Csx, tinman homolog low affinity sites
V$CDXF/CDX2.01	Cdx-2 mammalian caudal related intestinal transcr. factor
V$BRNF/BRN3.01	POU transcription factor Brn-3
V$TBPF/TATA.02	Mammalian C-type LTR TATA box
V$FKHD/FREAC3.01	Fork head related activator-3 (FOXC1)
V$OCT1/OCT1.02	Octamer-binding factor 1
V$CART/CART1.01	Cart-1 (cartilage homeoprotein 1)
V$PDX1/PDX1.01	Pdx1 (IDX 1/IPF1) pancreatic and intestinal homeodomain TF
V$PARF/DBP.01	Albumin D-box binding protein
V$GATA/GATA3.02	GATA-binding factor 3
V$VBPF/VBP.01	PAR-type chicken vitellogenin promoter-binding protein
V$AP4R/TAL1ALPHAE47.01	Tal-1alpha/E47 heterodimer
V$RP58/RP58.01	Zinc finger protein RP58 (ZNF238), associated preferentially with heterochromatin
V$COMP/COMP1.01	COMP1, cooperates with myogenic proteins in multicomponent complex
V$CLOX/CLOX.01	Clox
V$TBPF/ATATA.01	Avian C-type LTR TATA box
V$PBXC/PBX1_MEIS1.02	Binding site for a Pbx1/Meis1 heterodimer
V$PBXF/PBX1.01	Homeo domain factor Pbx-1

Family/matrix	Further Information
V$IRFF/IRF1.01	Interferon regulatory factor 1
V$TEAF/TEF1.01	TEF-1 related muscle factor

**matches are listed in order of occurrence in the corresponding sequence

The number of consensus transcription factor binding sites present in the vector backbone (including the ampicillin resistance gene) was reduced from 224 in pGL3 to 40 in pGL4, and the number of promoter modules was reduced from 10 in pGL3 to 4 for pGL4, using databases, search programs and the like as described herein. Other modifications in pGL4 relative to pGL3 include the removal of the f1 origin of replication and the redesign of the multiple cloning region.

MCS-1 to MCS-4 have the following sequences (SEQ ID Nos:76-79)

MCS-1
ACTAGTCGTCTCTCTTGAGAGACCGCGATCGCCACCATGATAAGTAA
GTAATATTAAATAAGTAAGGCCTGAGTGGCCCTCGAGCCAGCCTTGA
GTTGGTTGAGTCCAAGTCACGTCTGGAGATCTGGTACCTACGCGTGA
GCTCTACGTAGCTAGCGGCCTCGGCGGCCGAATTCTTGCGATCTAAG
TAAGCTTGGCATTCCGGTACTGTTGGTAAAGCCACCATGG

MCS-2
ACTAGTACGTCTCTCTTGAGAGACCGCGATCGCCACCATGATAAGTA
AGTAATATTAAATAAGTAAGGCCTGAGTGGCCCTCGAGTCCAGCCTT
GAGTTGGTTGAGTCCAAGTCACGTCTGGAGATCTGGTACCTTACGCGT
AGAGCTCTACGTAGCTAGCGGCCTCGGCGGCCGAATTCTTGCGATCT
AAGCTTGGCAATCCGGTACTGTTGGTAAAGCCACCATGG

MCS-3
ACTAGTACGTCTCTCTTGAGAGACCGCGATCGCATGCCTAGGTAGGT
AGTATTAGAGCATAGGTAGAGGCCTAAGTGGCCCTCGAGTCCAGCCT
TGAGTTGGTTGAGTCCAAGTCACGTCTGGAGATCTGGTACCTTACGCG
TATGAGCTCTACGTAGCTAGCGGCCTCGGCGGCCGAATTCTTGCGAT
CTAAGCTTGGCAATCCGGTACTGTTGGTAAAGCCACCATGG

MCS-4
ACTAGTACGTCTCTCTTGAGAGACCGCGATCGCCACCATGTCTAGGT
AGGTAGTAAACGAAAGGGCTTAAAGGCCTAAGTGGCCCTCGAGTCCA
GCCTTGAGTTGGTTGAGTCCAAGTCACGTTTGGAGATCTGGTACCTTA

CGCGTATGAGCTCTACGTAGCTAGCGGCCTCGGCGGCCGAATTCTTG
CGATCTAAGCTTGGCAATCCGGTACTGTTGGTAAAGCCACCATGG

bla has the following sequence:

ATGAGTATTCAACATTTCCGTGTCGCCCTTATTCCCTTTTTTGCGGCAT
TTTGCCTTCCTGTTTTTGCTCACCCAGAAACGCTGGTGAAAGTAAAAG
ATGCTGAAGATCAGTTGGGTGCACGAGTGGGTTACATCGAACTGGAT
CTCAACAGCGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAACGTTT
TCCAATGATGAGCACTTTTAAAGTTCTGCTATGTGGCGCGGTATTATC
CCGTATTGACGCCGGGCAAGAGCAACTCGGTCGCCGCATACACTATT
CTCAGAATGACTTGGTTGAGTACTCACCAGTCACAGAAAAGCATCTT
ACGGATGGCATGACAGTAAGAGAATTATGCAGTGCTGCCATAACCAT
GAGTGATAACACTGCGGCCAACTTACTTCTGACAACGATCGGAGGAC
CGAAGGAGCTAACCGCTTTTTTGCACAACATGGGGGATCATGTAACT
CGCCTTGATCGTTGGGAACCGGAGCTGAATGAAGCCATACCAAACGA
CGAGCGTGACACCACGATGCCTGTAGCAATGGCAACAACGTTGCGCA
AACTATTAACTGGCGAACTACTTACTCTAGCTTCCCGGCAACAATTAA
TAGACTGGATGGAGGCGGATAAAGTTGCAGGACCACTTCTGCGCTCG
GCCCTTCCGGCTGGCTGGTTTATTGCTGATAAATCTGGAGCCGGTGAG
CGTGGGTCTCGCGGTATCATTGCAGCACTGGGGCCAGATGGTAAGCC
CTCCCGTATCGTAGTTATCTACACGACGGGGAGTCAGGCAACTATGG
ATGAACGAAATAGACAGATCGCTGAGATAGGTGCCTCACTGATTAAG
CATTGGTAA (SEQ ID NO:41).

bla-1 to bla-5 have the following sequences (SEQ ID Nos:80-84):

bla-1

ACTAGTAACCCTGATAAATGCTTCAATAATATTGAAAAAGGAAGAGT
ATGAGTATTCAACATTTCCGTGTCGCCCTTATTCCCTTTTTTGCGGCAT
TTTGCCTTCCTGTTTTTGCTCACCCAGAAACGCTGGTGAAAGTAAAAG
ATGCTGAAGATCAGTTGGGTGCACGAGTGGGTTACATCGAACTGGAT
CTCAACAGCGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAACGTTT
TCCAATGATGAGCACTTTTAAAGTTCTGCTATGTGGCGCGGTATTATC
CCGTATTGACGCCGGGCAAGAGCAACTCGGTCGCCGCATACACTATT
CTCAGAATGACTTGGTTGAGTACTCACCAGTCACAGAAAAGCATCTT
ACGGATGGCATGACAGTAAGAGAATTATGCAGTGCTGCCATAACCAT
GAGTGATAACACCGCGGCCAACTTACTTCTGACAACGATCGGAGGAC
CGAAGGAGCTAACCGCTTTTTTGCACAACATGGGGGATCATGTAACT
CGCCTTGATCGTTGGGAACCGGAGCTGAATGAAGCCATACCAAACGA
CGAGCGTGACACCACGATGCCTGTAGCAATGGCAACAACGTTGCGCA

AACTATTAACTGGCGAACTACTTACTCTAGCTTCCCGGCAACAATTAA
TAGACTGGATGGAGGCGGATAAAGTTGCAGGACCACTTCTGCGCTCG
GCCCTTCCGGCTGGCTGGTTTATTGCTGATAAATCTGGAGCCGGTGAG
CGTGGCTCTCGCGGTATCATTGCAGCACTGGGGCCAGATGGTAAGCC
CTCCCGTATCGTAGTTATCTACACGACGGGGAGTCAGGCAACTATGG
ATGAACGAAATAGACAGATCGCTGAGATAGGTGCCTCACTGATTAAG
CATTGGTAACCACTGCAGTGGTTTTCCTTTTGCGGCCGC

bla-2

ACTAGTAACCCTGATAAATGCTGCAAACATATTGAAAAAGGAAGAGT
ATGAGTATTCAACATTTCCGTGTCGCACTCATTCCCTTCTTTGCGGCA
TTTTGCTTGCCTGTTTTTGCACACCCCGAAACGCTGGTGAAAGTAAAA
GATGCTGAAGATCAACTGGGTGCACGAGTGGGCTATATCGAACTGGA
TCTCAATAGCGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAACGTTT
TCCAATGATGAGCACTTTTAAAGTTCTGCTATGTGGCGCGGTATTATC
CCGTATTGACGCCGGGCAAGAGCAGCTCGGTCGCCGCATACACTACT
CACAGAACGACTTGGTTGAGTACTCGCCGGTCACGGAAAAGCATCTT
ACGGATGGCATGACAGTAAGAGAATTGTGTAGTGCTGCCATAACCAT
GAGTGATAACACCGCGGCCAACTTACTTCTGACAACGATCGGAGGCC
CTAAGGAGCTGACCGCATTTTTGCACAACATGGGGGATCATGTAACC
CGGCTTGATCGTTGGGAACCGGAGCTGAACGAAGCCATACCGAACGA
CGAGCGTGACACCACGATGCCTGTAGCAATGGCAACAACGTTGCGCA
AACTACTCACTGGCGAACTTCTCACTCTAGCATCACGACAGCAACTC
ATAGACTGGATGGAGGCGGATAAAGTTGCAGGACCACTTCTGCGCTC
GGCCCTTCCGGCTGGCTGGTTTATAGCTGATAAATCCGGTGCCGGTG
AACGCGGCTCTCGCGGGATCATTGCTGCGCTGGGGCCAGATGGTAAG
CCCTCACGAATCGTAGTTATCTACACGACGGGGAGTCAGGCAACTAT
GGATGAACGAAATAGACAGATCGCTGAGATAGGTGCCTCACTGATCA
AGCACTGGTAGCCACTGCAGTGGTTTAGCTTTTGCGGCCGC

bla-3

ACTAGTAACCCTGACAAATGCTGCAAACATATTGAAAAAGGAAGAGT
ATGAGCATCCAACATTTTCGTGTCGCACTCATTCCCTTCTTTGCGGCA
TTTTGCTTGCCTGTTTTTGCACACCCCGAAACGCTGGTGAAAGTAAAA
GATGCTGAAGATCAACTGGGTGCAAGAGTGGGCTATATCGAACTGGA
TCTCAATAGCGGCAAGATCCTTGAGTCTTTTCGCCCCGAAGAACGTTT
TCCGATGATGAGCACTTTTAAAGTTCTGCTATGTGGCGCGGTGTTGTC
CCGTATAGACGCCGGGCAAGAGCAGCTTGGTCGCCGTATACACTACT
CACAAAACGACTTGGTTGAGTACTCGCCGGTCACGGAAAAGCATCTT
ACGGATGGCATGACGGTAAGAGAATTGTGTAGTGCTGCCATTACCAT
GAGCGACAATACCGCGGCCAACTTACTTCTGACAACGATCGGAGGCC
CTAAGGAGCTGACCGCATTTTTGCACAACATGGGGGATCATGTAACC
CGGCTTGACCGCTGGGAACCGGAGCTGAACGAAGCCATACCGAACG
ACGAGCGTGACACCACGATGCCTGTAGCAATGGCAACAACGTTGCGG
AAACTACTCACTGGCGAACTTCTCACTCTAGCATCACGACAGCAGCT
CATAGACTGGATGGAGGCGGACAAAGTAGCAGGACCACTTCTTCGCT
CGGCCCTCCCTGCTGGCTGGTTCATTGCTGATAAATCCGGTGCCGGTG
AACGCGGCTCTCGCGGGATCATTGCTGCGCTGGGGCCTGATGGTAAG
CCCTCACGAATCGTAGTAATCTACACGACGGGGAGTCAGGCCACTAT

GGACGAACGAAATAGACAGATCGCTGAGATCGGTGCCTCACTGATCA
AGCACTGGTAACCACTGCAGTGGTTTAGCATTTGCGGCCGC

bla-4

ACTAGTAACCCTGACAAATGCTGCAAACATATTGAAAAAGGAAGAGT
ATGAGCATCCAACATTTTCGTGTCGCACTCATTCCCTTCTTTGCGGCA
TTTTGCTTGCCTGTTTTTGCACACCCCGAAACGCTGGTGAAAGTAAAA
GATGCTGAAGATCAACTGGGTGCAAGAGTGGGCTATATCGAACTGGA
TCTCAATAGCGGCAAGATCCTTGAGTCTTTCCGCCCCGAAGAACGTTT
TCCGATGATGAGCACTTTCAAAGTACTGCTATGTGGCGCGGTGTTGTC
CCGTATAGACGCCGGGCAAGAGCAGCTTGGTCGCCGTATACACTACT
CACAAAACGACTTGGTTGAGTACTCGCCGGTCACGGAAAAGCATCTT
ACGGATGGCATGACGGTAAGAGAATTGTGTAGTGCTGCCATTACCAT
GAGCGATAATACCGCGGCCAACTTACTTCTGACAACGATCGGAGGCC
CTAAGGAGCTGACCGCATTTTTGCACAACATGGGTGATCATGTGACC
CGGCTTGACCGCTGGGAACCGGAGCTGAACGAAGCCATACCGAACG
ACGAGCGTGACACCACGATGCCTGTAGCAATGGCAACAACTCTTCGG
AAACTACTCACTGGCGAACTTCTCACTCTAGCATCACGACAGCAGCT
CATAGACTGGATGGAGGCGGACAAAGTAGCAGGACCACTTCTTCGCT
CGGCCCTCCCTGCTGGCTGGTTCATTGCTGATAAATCTGGAGCCGGTG
AGCGTGGCTCTCGCGGTATCATTGCTGCGCTGGGGCCTGATGGTAAG
CCCTCACGAATCGTAGTAATCTACACGACGGGGAGTCAGGCCACTAT
GGACGAACGAAATAGACAGATCGCTGAGATCGGTGCCTCACTGATCA
AGCACTGGTAACCACTGCAGTGGTTTAGCATTTGCGGCCGC

bla-5

ACTAGTAACCCTGACAAATGCTGCAAACATATTGAAAAAGGAAGAGT
ATGAGCATCCAACATTTTCGTGTCGCACTCATTCCCTTCTTTGCGGCA
TTTTGCTTGCCTGTTTTTGCACACCCCGAAACGCTGGTGAAAGTAAAA
GATGCTGAAGATCAACTGGGTGCAAGAGTGGGCTATATCGAACTGGA
TCTCAATAGCGGCAAGATCCTTGAGTCTTTCCGCCCCGAAGAACGAT
TCCCGATGATGAGCACTTTCAAAGTACTGCTATGTGGCGCGGTGTTGT
CCCGTATAGACGCCGGGCAAGAGCAGCTTGGTCGCCGTATACACTAC
TCACAAAACGACTTGGTTGAGTACTCGCCGGTCACGGAAAAGCATCT
TACGGATGGCATGACGGTAAGAGAATTGTGTAGTGCTGCCATTACCA
TGAGCGATAATACCGCGGCCAACTTACTTCTGACAACGATCGGAGGC
CCTAAGGAGCTGACCGCATTTTTGCACAACATGGGTGATCATGTGAC
CCGGCTTGACCGCTGGGAACCGGAGCTGAACGAAGCCATACCGAAC
GACGAGCGTGATACCACGATGCCAGTAGCAATGGCCACAACTCTTCG
GAAACTACTCACTGGCGAACTTCTCACTCTAGCATCACGACAGCAGC
TCATAGACTGGATGGAGGCGGACAAAGTAGCAGGACCACTTCTTCGC
TCGGCCCTCCCTGCTGGCTGGTTCATTGCTGACAAATCCGGTGCCGGT
GAACGCGGCTCTCGCGGCATCATTGCTGCGCTGGGGCCTGATGGTAA
GCCCTCACGAATCGTAGTAATCTACACGACGGGGAGTCAGGCCACTA
TGGACGAACGAAATAGACAGATCGCTGAGATCGGTGCCTCACTGATC
AAGCACTGGTAACCACTGCAGTGGTTTAGCATTTGCGGCCGCNNN.

Table 30

Pairwise identity of different *bla* gene versions

	bla	bla-1	bla-2	bla-3	bla-4	bla-5	bla in pGL4 (SEQ ID NO:74)
bla	--	99	93	90	89	88	87
bla-1		--	94	90	90	89	88
bla-2			--	96	94	94	93
bla-3				--	98	98	97
bla-4					--	99	97
bla-5						--	98

note: sequence "bla" is bla gene from pGL3-Basic; ClustalW (Slow/Accurate, IUB); sequence comparisons were of ORF only

SpeI-NcoI ver2 start has the following sequence:
ACTAGTACGTCTCTCAAGGATAAGTAAGTAATATTAAGGTACGGGAG
GTACTTGGAGCGGCCGCAATAAAATATCTTTATTTTCATTACATCTGT
GTGTTGGTTTTTTGTGTGAATCGATAGTACTAACATACGCTCTCCATC
AAAACAAAACGAAACAAAACAAACTAGCAAAATAGGCTGTCCCCAG
TGCAAGTGCAGGTGCCAGAACATTTCTCTGGCCTAAGTGGCCGGTAC
CGAGCTCGCTAGCCTCGAGGATATCAGATCTGGCCTCGGCGGCCAAG
CTTGGCAATCCGGTACTGTTGGTAAAGCCACCATGG (SEQ ID NO:48); and

SpeI-NcoI-Ver2 has the following sequence:
ACTAGTACGTCTCTCAAGGATAAGTAAGTAATATTAAGGTACGGGAG
GTATTGGACAGGCCGCAATAAAATATCTTTATTTTCATTACATCTGTG
TGTTGGTTTTTTGTGTGAATCGATAGTACTAACATACGCTCTCCATCA
AAACAAAACGAAACAAAACAAACTAGCAAAATAGGCTGTCCCCAGT
GCAAGTGCAGGTGCCAGAACATTTCTCTGGCCTAACTGGCCGGTACC
TGAGCTCGCTAGCCTCGAGGATATCAAGATCTGGCCTCGGCGGCCAA
GCTTGGCAATCCGGTACTGTTGGTAAAGCCACCATGG (SEQ ID NO:49)

pGL4 related sequences include (SEQ ID Nos.95-97):

pGL4B-4NN

GCGGCCGCAAATGCTAAACCACTGCAGTGGTTACCAGTGCTTGATCA
GTGAGGCACCGATCTCAGCGATCTGTCTATTTCGTTCGTCCATAGTGG
CCTGACTCCCCGTCGTGTAGATTACTACGATTCGTGAGGGCTTACCAT
CAGGCCCCAGCGCAGCAATGATGCCGCGAGAGCCGCGTTCACCGGCA

CCGGATTTGTCAGCAATGAACCAGCCAGCAGGGAGGGCCGAGCGAA
GAAGTGGTCCTGCTACTTTGTCCGCCTCCATCCAGTCTATGAGCTGCT
GTCGTGATGCTAGAGTGAGAAGTTCGCCAGTGAGTAGTTTCCGAAGA
GTTGTGGCCATTGCTACTGGCATCGTGGTATCACGCTCGTCGTTCGGT
ATGGCTTCGTTCAGCTCCGGTTCCCAGCGGTCAAGCCGGGTCACATG
ATCACCCATGTTGTGCAAAAATGCGGTCAGCTCCTTAGGGCCTCCGA
TCGTTGTCAGAAGTAAGTTGGCCGCGGTATTATCGCTCATGGTAATGG
CAGCACTACACAATTCTCTTACCGTCATGCCATCCGTAAGATGCTTTT
CCGTGACCGGCGAGTACTCAACCAAGTCGTTTTGTGAGTAGTGTATA
CGGCGACCAAGCTGCTCTTGCCCGGCGTCTATACGGGACAACACCGC
GCCACATAGCAGTACTTTGAAAGTGCTCATCATCGGGAATCGTTCTTC
GGGGCGGAAAGACTCAAGGATCTTGCCGCTATTGAGATCCAGTTCGA
TATAGCCCACTCTTGCACCCAGTTGATCTTCAGCATCTTTTACTTTCAC
CAGCGTTTCGGGGTGTGCAAAAACAGGCAAGCAAAATGCCGCAAAG
AAGGGAATGAGTGCGACACGAAAATGTTGGATGCTCATACTCTTCCT
TTTTCAATATGTTTGCAGCATTTGTCAGGGTTACTAGTACGTCTCTCTT
GAGAGACCGCGATCGCCACCATGTCTAGGTAGGTAGTAAACGAAAG
GGCTTAAAGGCCTAAGTGGCCCTCGAGTCCAGCCTTGAGTTGGTTGA
GTCCAAGTCACGTTTGGAGATCTGGTACCTTACGCGTATGAGCTCTAC
GTAGCTAGCGGCCTCGGCGGCCGAATTCTTGCGATCTAAGCTTGGCA
ATCCGGTACTGTTGGTAAAGCCACCATGG

pGL4B-4NN1
gcggccgcaaatgctaaaccactgcagtggttaccagtgcttgatcagtgaggcaccgatctcagcgatctgtctatt
tcgttcgtccatagtggcctgactccccgtcgtgtagattactacgattcgtgagggcttaccatcaggccccagcgc
agcaatgatgccgcgagagccgcgttcaccggcccccgatttgtcagcaatgaaccagccagcagggagggccg
agcgaagaagtggtcctgctactttgtccgcctccatccagtctatgagctgctgtcgtgatgctagagtaagaagttc
gccagtgagtagtttccgaagagttgtggccattgctactggcatcgtggtatcacgctcgtcgttcggtatggcttcgt
tcaactccggttcccagcggtcaagccgggtcacatgatcacccatgttgtgcaaaaatgcggtcagctccttaggg
cctccgatcgttgtcagaagtaagttggccgcggtgttgtcgctcatggtaatggcagcactacacaattctcttaccgt
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agctgctcttgcccggcgtctatacgggacaacaccgcgccacatagcagtactttgaaagtgctcatcatcgggaa
tcgttcttcggggcggaaagactcaaggatcttgccgctattgagatccagttcgatatagcccactcttgcacccagt
tgatcttcagcatcttttactttcaccagcgtttcggggtgtgcaaaaacaggcaagcaaaatgccgcaaagaaggga
atgagtgcgacacgaaaatgttggatgctcatactcttccttttcaatatgtttgcagcatttgtcagggttactagtacg
tctctcttgagagaccgcgatcgccaccatgtctaggtaggtagtaaacgaaagggcttaaaggcctaagtggccct
cgagtccagccttgagttggttgagtccaagtcacgtttggagatctggtaccttacgcgtatgagctctacgtagcta
gcggcctcggcggccgaattcttgcgttcgaagcttggcaatccggtactgttggtaaagccaccatgg; and

pGL4B-4NN2
GCGGCCGCAAATGCTAAACCACTGCAGTGGTTACCAGTGCTTGATCA
GTGAGGCACCGATCTCAGCGATCTGCCTATTTCGTTCGTCCATAGTGG
CCTGACTCCCCGTCGTGTAGATCACTACGATTCGTGAGGGCTTACCAT
CAGGCCCCAGCGCAGCAATGATGCCGCGAGAGCCGCGTTCACCGGCC
CCCGATTTGTCAGCAATGAACCAGCCAGCAGGGAGGGCCGAGCGAA
GAAGTGGTCCTGCTACTTTGTCCGCCTCCATCCAGTCTATGAGCTGCT
GTCGTGATGCTAGAGTAAGAAGTTCGCCAGTGAGTAGTTTCCGAAGA
GTTGTGGCCATTGCTACTGGCATCGTGGTATCACGCTCGTCGTTCGGT
ATGGCTTCGTTCAACTCTGGTTCCCAGCGGTCAAGCCGGGTCACATG

ATCACCCATGTTGTGCAAAAATGCGGTCAGCTCCTTAGGGCCTCCGA
TCGTTGTCAGAAGTAAGTTGGCCGCGGTGTTGTCGCTCATGGTAATGG
CAGCACTACACAATTCTCTTACCGTCATGCCATCCGTAAGATGCTTTT
CCGTGACCGGCGAGTACTCAACCAAGTCGTTTTGTGAGTAGTGTATA
CGGCGACCAAGCTGCTCTTGCCCGGCGTCTATACGGGACAACACCGC
GCCACATAGCAGTACTTTGAAAGTGCTCATCATCGGGAATCGTTCTTC
GGGGCGGAAAGACTCAAGGATCTTGCCGCTATTGAGATCCAGTTCGA
TATAGCCCACTCTTGCACCCAGTTGATCTTCAGCATCTTTTACTTTCAC
CAGCGTTTCGGGGTGTGCAAAAACAGGCAAGCAAAATGCCGCAAAG
AAGGGAATGAGTGCGACACGAAAATGTTGGATGCTCATACTCTTCCT
TTTTCAATATGTTTGCAGCATTTGTCAGGGTTACTAGTACGTCTCTCTT
GAGAGACCGCGATCGCCACCATGTCTAGGTAGGTAGTAAACGAAAG
GGCTTAAAGGCCTAAGTGGCCCTCGAGTCCAGCCTTGAGTTGGTTGA
GTCCAAGTCACGTTTGGAGATCTGGTACCTTACGCGTATGAGCTCTAC
GTAGCTAGCGGCCTCGGCGGCCGAATTCTTGCGTTCGAAGCTTGGCA
ATCCGGTACTGTTGGTAAAGCCACCATGG,

as well as

pGL4B-4NN3:

GCGGCCGCAAATGCTAAACCACTGCAGTGGTTACCAGTGCTTGATCA
GTGAGGCACCGATCTCAGCGATCTGCCTATTTCGTTCGTCCATAGTGG
CCTGACTCCCCGTCGTGTAGATCACTACGATTCGTGAGGGCTTACCAT
CAGGCCCCAGCGCAGCAATGATGCCGCGAGAGCCGCGTTCACCGGCC
CCCGATTTGTCAGCAATGAACCAGCCAGCAGGGAGGGCCGAGCGAA
GAAGTGGTCCTGCTACTTTGTCCGCCTCCATCCAGTCTATGAGCTGCT
GTCGTGATGCTAGAGTAAGAAGTTCGCCAGTGAGTAGTTTCCGAAGA
GTTGTGGCCATTGCTACTGGCATCGTGGTATCACGCTCGTCGTTCGGT
ATGGCTTCGTTCAACTCTGGTTCCCAGCGGTCAAGCCGGGTCACATG
ATCACCCATATTATGAAGAAATGCAGTCAGCTCCTTAGGGCCTCCGA
TCGTTGTCAGAAGTAAGTTGGCCGCGGTGTTGTCGCTCATGGTAATGG
CAGCACTACACAATTCTCTTACCGTCATGCCATCCGTAAGATGCTTTT
CCGTGACCGGCGAGTACTCAACCAAGTCGTTTTGTGAGTAGTGTATA
CGGCGACCAAGCTGCTCTTGCCCGGCGTCTATACGGGACAACACCGC
GCCACATAGCAGTACTTTGAAAGTGCTCATCATCGGGAATCGTTCTTC
GGGGCGGAAAGACTCAAGGATCTTGCCGCTATTGAGATCCAGTTCGA
TATAGCCCACTCTTGCACCCAGTTGATCTTCAGCATCTTTTACTTTCAC
CAGCGTTTCGGGGTGTGCAAAAACAGGCAAGCAAAATGCCGCAAAG
AAGGGAATGAGTGCGACACGAAAATGTTGGATGCTCATACTCTTCCT
TTTTCAATATGTTTGCAGCATTTGTCAGGGTTACTAGTACGTCTCTCTT
GAGAGACCGCGATCGCCACCATGTCTAGGTAGGTAGTAAACGAAAG
GGCTTAAAGGCCTAAGTGGCCCTCGAGTCCAGCCTTGAGTTGGTTGA
GTCCAAGTCACGTTTGGAGATCTGGTACCTTACGCGTATGAGGGTTG
AGTCCAAGTCACGTTTGGAGATCTGGTACCTTACGCGTATGAGCTCTA
CGTAGCTAGCGGCCTCGGCGGCCGAATTCTTGCGTTCGAAGCTTGGC
AATCCGGTACTGTTGGTAAAGCCACCATGG (SEQ ID NO:45)

pGL4NN from Blue Heron:

GCGGCCGCAAATGCTAAACCACTGCAGTGGTTACCAGTGCTTGATCA

GTGAGGCACCGATCTCAGCGATCTGCCTATTTCGTTCGTCCATAGTGG
CCTGACTCCCCGTCGTGTAGATCACTACGATTCGTGAGGGCTTACCAT
CAGGCCCCAGCGCAGCAATGATGCCGCGAGAGCCGCGTTCACCGGCC
CCCGATTTGTCAGCAATGAACCAGCCAGCAGGGAGGGCCGAGCGAA
GAAGTGGTCCTGCTACTTTGTCCGCCTCCATCCAGTCTATGAGCTGCT
GTCGTGATGCTAGAGTAAGAAGTTCGCCAGTGAGTAGTTTCCGAAGA
GTTGTGGCCATTGCTACTGGCATCGTGGTATCACGCTCGTCGTTCGGT
ATGGCTTCGTTCAACTCTGGTTCCCAGCGGTCAAGCCGGGTCACATG
ATCACCCATATTATGAAGAAATGCAGTCAGCTCCTTAGGGCCTCCGA
TCGTTGTCAGAAGTAAGTTGGCCGCGGTGTTGTCGCTCATGGTAATGG
CAGCACTACACAATTCTCTTACCGTCATGCCATCCGTAAGATGCTTTT
CCGTGACCGGCGAGTACTCAACCAAGTCGTTTTGTGAGTAGTGTATA
CGGCGACCAAGCTGCTCTTGCCCGGCGTCTATACGGGACAACACCGC
GCCACATAGCAGTACTTTGAAAGTGCTCATCATCGGGAATCGTTCTTC
GGGGCGGAAAGACTCAAGGATCTTGCCGCTATTGAGATCCAGTTCGA
TATAGCCCACTCTTGCACCCAGTTGATCTTCAGCATCTTTTACTTTCAC
CAGCGTTTCGGGGTGTGCAAAAACAGGCAAGCAAAATGCCGCAAAG
AAGGGAATGAGTGCGACACGAAAATGTTGGATGCTCATACTCTTCCT
TTTTCAATATGTTTGCAGCATTTGTCAGGGTTACTAGTACGTCTCTCA
AGAGATTTGTGCATACACAGTGACTCATACTTTCACCAATACTTTGCA
TTTTGGATAAATACTAGACAACTTTAGAAGTGAATTATTTATGAGGTT
GTCTTAAAATTAAAAATTACAAAGTAATAAATCACATTGTAATGTATT
TTGTGTGATACCCAGAGGTTTAAGGCAACCTATTACTCTTATGCTCCT
GAAGTCCACAATTCACAGTCCTGAACTATAATCTTATCTTTGTGATTG
CTGAGCAAATTTGCAGTATAATTTCAGTGCTTTTAAATTTTGTCCTGC
TTACTATTTTCCTTTTTTATTTGGGTTTGATATGCGTGCACAGAATGGG
GCTTCTATTAAAATATTCTTGAGAGACCGCGATCGCCACCATGTCTAG
GTAGGTAGTAAACGAAAGGGCTTAAAGGCCTAAGTGGCCCTCGAGTC
CAGCCTTGAGTTGGTTGAGTCCAAGTCACGTTTGGAGATCTGGTACCT
TACGCGTATGAGCTCTACGTAGCTAGCGGCCTCGGCGGCCGAATTCT
TGCGTTCGAAGCTTGGCAATCCGGTACTGTTGGTAAAGCCACCATGG
(SEQ ID NO:46),

pGL4 with promoter changes:

GCGGCCGCAAATGCTAAACCACTGCAGTGGTTACCAGTGCTTGATCA
GTGAGGCACCGATCTCAGCGATCTGCCTATTTCGTTCGTCCATAGTGG
CCTGACTCCCCGTCGTGTAGATCACTACGATTCGTGAGGGCTTACCAT
CAGGCCCCAGCGCAGCAATGATGCCGCGAGAGCCGCGTTCACCGGCC
CCCGATTTGTCAGCAATGAACCAGCCAGCAGGGAGGGCCGAGCGAA
GAAGTGGTCCTGCTACTTTGTCCGCCTCCATCCAGTCTATGAGCTGCT
GTCGTGATGCTAGAGTAAGAAGTTCGCCAGTGAGTAGTTTCCGAAGA
GTTGTGGCCATTGCTACTGGCATCGTGGTATCACGCTCGTCGTTCGGT
ATGGCTTCGTTCAACTCTGGTTCCCAGCGGTCAAGCCGGGTCACATG
ATCACCCATATTATGAAGAAATGCAGTCAGCTCCTTAGGGCCTCCGA

TCGTTGTCAGAAGTAAGTTGGCCGCGGTGTTGTCGCTCATGGTAATGG
CAGCACTACACAATTCTCTTACCGTCATGCCATCCGTAAGATGCTTTT
CCGTGACCGGCGAGTACTCAACCAAGTCGTTTTGTGAGTAGTGTATA
CGGCGACCAAGCTGCTCTTGCCCGGCGTCTATACGGGACAACACCGC
GCCACATAGCAGTACTTTGAAAGTGCTCATCATCGGGAATCGTTCTTC
GGGGCGGAAAGACTCAAGGATCTTGCCGCTATTGAGATCCAGTTCGA
TATAGCCCACTCTTGCACCCAGTTGATCTTCAGCATCTTTTACTTTCAC
CAGCGTTTCGGGGTGTGCAAAAACAGGCAAGCAAAATGCCGCAAAG
AAGGGAATGAGTGCGACACGAAAATGTTGGATGCTCATACTCGTCCT
TTTTCAATATTATTGAAGCATTTATCAGGGTTACTAGTACGTCTCTCA
AGAGATTTGTGCATACACAGTGACTCATACTTTCACCAATACTTTGCA
TTTTGGATAAATACTAGACAACTTTAGAAGTGAATTATTTATGAGGTT
GTCTTAAAATTAAAAATTACAAAGTAATAAATCACATTGTAATGTATT
TTGTGTGATACCCAGAGGTTTAAGGCAACCTATTACTCTTAT (SEQ ID NO:47),

A hygromycin gene in a pGL4 vector:

Atgaagaagcccgaactcaccgctaccagcgttgaaaaatttctcatcgagaagttcgacagtgtgagcgacctgat
gcagttgtcggagggcgaagagagccgagccttcagcttcgatgtcggcggacgcggctatgtactgcgggtgaa
tagctgcgctgatggcttctacaaagaccgctacgtgtaccgccacttcgccagcgctgcactacccatccccgaag
tgttggacatcggcgagttcagcgagagcctgacatactgcatcagtagacgcgcccaaggcgttactctccaaga
cctccccgaaacagagctgcctgctgtgttacagcctgtcgccgaagctatggatgctattgccgccgccgacctca
gtcaaaccagcggcttcggcccattcgggccccaaggcatcggccagtacacaacctggcgggatttcatttgcgc
cattgctgatccccatgtctaccactggcagaccgtgatggacgacaccgtgtccgccagcgtagctcaagccctgg
acgaactgatgctgtgggccgaagactgtcccgaggtgcgccacctcgtccatgccgacttcggcagcaacaacgt
cctgaccgacaacggccgcatcaccgccgtaatcgactggtccgaagctatgttcggggacagtcagtacgaggtg
gccaacatcttcttctggcggccctggctggcttgcatggagcagcagactcgctacttcgagcgccggcatcccga
gctggccggcagccctcgtctgcgagcctacatgctgcgcatcggcctggatcagctctaccagagcctcgtggac
ggcaacttcgacgatgctgcctgggctcaaggccgctgcgatgccatcgtccgcagcggggccggcaccgtcggt
cgcacacaaatcgctcgccggagcgcagccgtatggaccgacggctgcgtcgaggtgctggccgacagcggca
accgccggcccagtacacgaccgcgcgctaaggaggtaggtcgagtttaa (SEQ ID NO:88),

pGL4.10

ggcctaactggccggtacctgagctcgctagcctcgaggatatcaagatctggcctcggcggccaagcttggcaat
ccggtactgttggtaaagccaccatggaagatgccaaaaacattaagaagggcccagcgccattctacccactcga
agacgggaccgccggcgagcagctgcacaaagccatgaagcgctacgccctggtgcccggcaccatcgccttta
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tgccctgttcatcggtgtggctgtggccccagctaacgacatctacaacgagcgcgagctgctgaacagcatgggc

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catttgccacccggcttcaacgagtacgacttcgtgcccgagagcttcgacccgggacaaaaccatcgccctgatcat
gaacagtagtggcagtaccggattgcccaagggcgtagccctaccgcaccgcaccgcttgtgtccgattcagtcat
gcccgcgacccccatcttcggcaaccagatcatccccgacaccgctatcctcagcgtggtgccatttcaccacggctt
cggcatgttcaccacgctgggctacttgatctgcggctttcgggtcgtgctcatgtaccgcttcgaggaggagctattc
ttgcgcagcttgcaagactataagattcaatctgccctgctggtgcccacactatttagcttcttcgctaagagcactct
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aggccgtggccaaacgcttccacctaccaggcatccgccagggctacggcctgacagaaacaaccagcgccattc
tgatcacccccgaaggggacgacaagcctggcgcagtaggcaaggtggtgcccttcttcgaggctaaggtggtgg
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gctacgttaacaaccccgaggctacaaacgctctcatcgacaaggacggctggctgcacagcggcgacatcgcct
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cccagccgaactggagagcatcctgctgcaacaccccaacatcttcgacgccggggtcgccggcctgcccgacg
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gactatgtggccagccaggttacaaccgccaagaagctgcgcggtggtgttgtgttcgtggacgaggtgcctaaag
gactgaccggcaagttggacgcccgcaagatccgcgagattctcattaaggccaagaagggcggcaagatcgcc
gtgtaataattctagagtcggggcggccggccgcttcgagcagacatgataagatacattgatgagtttggacaaac
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aatcgacgctcaagtcagaggtggcgaaacccgacaggactataaagataccaggcgtttccccctggaagctccc
tcgtgcgctctcctgttccgaccctgccgcttaccggatacctgtccgcctttctcccttcgggaagcgtggcgctttct
catagctcacgctgtaggtatctcagttcggtgtaggtcgttcgctccaagctgggctgtgtgcacgaacccccgttc
agcccgaccgctgcgccttatccggtaactatcgtcttgagtccaacccggtaagacacgacttatcgccactggca
gcagccactggtaacaggattagcagagcgaggtatgtaggcggtgctacagagttcttgaagtggtggcctaacta
cggctacactagaagaacagtatttggtatctgcgctctgctgaagccagttaccttcggaaaaagagttggtagctct
tgatccggcaaacaaaccaccgctggtagcggtggttttttgtttgcaagcagcagattacgcgcagaaaaaaagg
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aacttggtctgacagcggccgcaaatgctaaaccactgcagtggttaccagtgcttgatcagtgaggcaccgatctc
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caggccccagcgcagcaatgatgccgcgagaggccgcgttcaccggcccccgatttgtcagcaatgaaccagcca
gcagggagggccgagcgaagaagtggtcctgctactttgtccgcctccatccagtctatgagctgctgtcgtgatgc
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caaaacaaactagcaaaataggctgtccccagtgcaagtgcaggtgccagaacatttctctaagtaatattaaggtac

gggaggtattggacaggccgcaataaaatatctttattttcattacatctgtgtgttggttttttgtgtgaatc (SEQ ID NO:89), and

pGL4.70

ggcctaactggccggtacctgagctcgctagcctcgaggatatcaagatctggcctcggcggccaagcttggcaat
ccggtactgttggtaaagccaccatggcttccaaggtgtacgaccccgagcaacgcaaacgcatgatcactgggcc
tcagtggtgggctcgctgcaagcaaatgaacgtgctggactccttcatcaactactatgattccgagaagcacgccg
agaacgccgtgatttttctgcatggtaacgctgcctccagctacctgtggaggcacgtcgtgcctcacatcgagcccg
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gacgtgatcgagtcctgggacgagtggcctgacatcgaggaggatatcgccctgatcaagagcgaagagggcga
gaaaatggtgcttgagaataacttcttcgtcgagaccatgctcccaagcaagatcatgcggaaactggagcctgagg
agttcgctgcctacctggagccattcaaggagaagggcgaggttagacggcctaccctctcctggcctcgcgagat
ccctctcgttaagggaggcaagcccgacgtcgtccagattgtccgcaactacaacgcctaccttcggccagcgac
gatctgcctaagatgttcatcgagtccgaccctgggttcttttccaacgctattgtcgagggagctaagaagttccctaa
caccgagttcgtgaaggtgaagggcctccacttcagccaggaggacgctccagatgaaatgggtaagtacatcaag
agcttcgtggagcgcgtgctgaagaacgagcagtaattctagagtcggggcggccggccgcttcgagcagacatg
ataagatacattgatgagtttggacaaaccacaactagaatgcagtgaaaaaaatgctttatttgtgaaatttgtgatgct
attgctttatttgtaaccattataagctgcaataaacaagttaacaacaacaattgcattcattttatgtttcaggttcaggg
ggaggtgtgggaggttttttaaagcaagtaaaacctctacaaatgtggtaaaatcgataaggatccgtcgaccgatgc
ccttgagagccttcaacccagtcagctccttccggtgggcgcggggcatgactatcgtcgccgcacttatgactgtct
tctttatcatgcaactcgtaggacaggtgccggcagcgctcttccgcttcctcgctcactgactcgctgcgctcggtcg
ttcggctgcggcgagcggtatcagctcactcaaaggcggtaatacggttatccacagaatcaggggataacgcagg
aaagaacatgtgagcaaaaggccagcaaaaggccaggaaccgtaaaaaggccgcgttgctggcgttttccatag
gctccgcccccctgacgagcatcacaaaaatcgacgctcaagtcagaggtggcgaaacccgacaggactataaag
ataccaggcgtttcccccctggaagctccctcgtgcgctctcctgttccgaccctgccgcttaccggatacctgtccgc
ctttctcccttcgggaagcgtggcgctttctcatagctcacgctgtaggtatctcagttcggtgtaggtcgttcgctcca
agctgggctgtgtgcacgaaccccccgttcagcccgaccgctgcgccttatccggtaactatcgtcttgagtccaac
ccggtaagacacgacttatcgccactggcagcagccactggtaacaggattagcagagcgaggtatgtaggcggt
gctacagagttcttgaagtggtggcctaactacggctacactagaagaacagtatttggtatctgcgctctgctgaagc
cagttaccttcggaaaaagagttggtagctcttgatccggcaaacaaaccaccgctggtagcggtggtttttttgtttgc
aagcagcagattacgcgcagaaaaaaaggatctcaagaagatcctttgatcttttctacggggtctgacgctcagtgg
aacgaaaactcacgttaagggattttggtcatgagattatcaaaaaggatcttcacctagatccttttaaattaaaaatga
agttttaaatcaatctaaagtatatatgagtaaacttggtctgacagcggccgcaaatgctaaaccactgcagtggttac
cagtgcttgatcagtgaggcaccgatctcagcgatctgcctatttcgttcgtccatagtggcctgactccccgtcgtgta
gatcactacgattcgtgagggcttaccatcaggccccagcgcagcaatgatgccgcgagagccgcgttcaccggc
ccccgatttgtcagcaatgaaccagccagcagggagggccgagcgaagaagtggtcctgctacttttgtccgcctcc
atccagtctatgagctgctgtcgtgatgctagagtaagaagttcgccagtgagtagtttccgaagagttgtggccattg
ctactggcatcgtggtatcacgctcgtcgttcggtatggcttcgttcaactctggttcccagcggtcaagccgggtcac
atgatcacccatattatgaagaaatgcagtcagctccttagggcctccgatcgttgtcagaagtaagttggccgcggt
gttgtcgctcatggtaatggcagcactacacaattctcttaccgtcatgccatccgtaagatgcttttccgtgaccggcg
agtactcaaccaagtcgttttgtgagtagtgtatacggcgaccaagctgctcttgcccggcgtctatacgggacaaca
ccgcgccacatagcagtactttgaaagtgctcatcatcgggaatcgttcttcggggcggaaagactcaaggatcttgc
cgctattgagatccagttcgatatagcccactcttgcacccagttgatcttcagcatcttttactttcaccagcgtttcggg
gtgtgcaaaaacaggcaagcaaaatgccgcaaagaagggaatgagtgcgacacgaaaatgttggatgctcatact
cgtccttttcaatattattgaagcatttatcagggttactagtacgtctctcaaggataagtaagtaatattaaggtacgg
gaggtattggacaggccgcaataaaatatctttattttcattacatctgtgtgttggttttttgtgtgaatcgatagtactaa

catacgctctccatcaaaacaaaacgaaacaaaacaaactagcaaaataggctgtccccagtgcaagtgcaggtgc
cagaacatttctct (SEQ ID NO:90).

The pGL4 backbone (*Not*I-*Nco*I) has the following sequence:
gcggccgcaaatgctaaaccactgcagtggttaccagtgcttgatcagtgaggcaccgatctcagcgatctgcctatt
tcgttcgtccatagtggcctgactccccgtcgtgtagatcactacgattcgtgagggcttaccatcaggccccagcgc
agcaatgatgccgcgagagccgcgttcaccggcccccgatttgtcagcaatgaaccagccagcagggagggccg
agcgaagaagtggtcctgctactttgtccgcctccatccagtctatgagctgctgtcgtgatgctagagtaagaagttc
gccagtgagtagtttccgaagagttgtggccattgctactggcatcgtggtatcacgctcgtcgttcggtatggcttcgt
tcaactctggttcccagcggtcaagccgggtcacatgatcacccatattatgaagaaatgcagtcagctccttagggc
ctccgatcgttgtcagaagtaagttggccgcggtgttgtcgctcatggtaatggcagcactacacaattctcttaccgtc
atgccatccgtaagatgcttttccgtgaccggcgagtactcaaccaagtcgttttgtgagtagtgtatacggcgaccaa
gctgctcttgcccggcgtctatacgggacaacaccgcgccacatagcagtactttgaaagtgctcatcatcgggaat
cgttcttcggggcggaaagactcaaggatcttgccgctattgagatccagttcgatatagcccactcttgcacccagtt
gatcttcagcatcttttactttcaccagcgtttcggggtgtgcaaaaacaggcaagcaaaatgccgcaaagaaggga
atgagtgcgacacgaaaatgttggatgctcatactcgtccttttcaatattattgaagcatttatcagggttactagtacg
tctctcaaggataagtaagtaatattaaggtacgggaggtattggacaggccgcaataaaatatctttatttcattacat
ctgtgtgttggttttttgtgtgaatcgatagtactaacatacgctctccatcaaaacaaaacgaaacaaaacaaactagc
aaaataggctgtccccagtgcaagtgcaggtgccagaacatttctctggcctaactggccggtacctgagctcgcta
gcctcgaggatatcaagatctggcctcggcggccaagcttggcaatccggtactgttggtaaagccaccatgg
(SEQ ID NO:74).

Example 10

Summary of Sequences Removed in Synthetic Genes

Search parameters:

TFBS searches were limited to vertebrate TF binding sites. Searches were performed by matrix family, *i.e.*, the results show only the best match from a family for each site. MatInspector default parameters were used for the core and matrix similarity values (core similarity = 0.75, matrix similarity = optimized), except for sequence MCS-1 (core similarity = 1.00, matrix similarity = optimized).

Promoter module searches included all available promoter modules (vertebrate and others) and were performed using default parameters (optimized threshold or 80% of maximum score).

Splice site searches were performed for splice acceptor or donor consensus sequences.

Table 31

Sequence	Matrix Library	TFBS (family matches)	Promoter modules	Splice sites (+ strand)
puro	(not applicable)	62	5	0
hpuro	(not applicable)	68	4	1
hpuro1	Ver 4.1 Feb 2004	4	2	1
hpuro2	Ver 4.1 Feb 2004	2	0	1
---	---	---	---	---
Neo	(not applicable)	53	0	No data
hneo	(not applicable)	61	2	3
hneo-1	Ver 3.1.2 Jun 2003	No data	No data	No data
hneo-2	Ver 3.1.2 Jun 2003	No data	No data	No data
hneo-3	Ver 3.1.2 Jun 2003	0	0	0
hneo-4	Ver 4.1 Feb 2004	7	1	0
hneo-5	Ver 4.1 Feb 2004	0	0	0
---	---	---	---	---
Hyg	(not applicable)	74	3	No data
hhyg	(not applicable)	94	4	6
hhyg-1	Ver 3.1.2 Jun 2003	No data	No data	No data
hhyg-2	Ver 3.1.2 Jun 2003	No data	No data	No data
hhyg-3	Ver 3.1.2 Jun 2003	3	0	0
hHygro	Ver 3.3 Aug 2003	5	0	0
hhyg-4	Ver 3.3 Aug 2003	4	0	0
---	---	---	---	---
Luc	(not applicable)	213	11	No data
Luc+	(not	189	7	No data

Sequence	Matrix Library	TFBS (family matches)	Promoter modules	Splice sites (+ strand)
	applicable)			
hluc+ver2A1	Ver 3.0 Nov 2002	110	7	6
hluc+ver2A2	Ver 3.0 Nov 2002	No data	No data	No data
hluc+ver2A3	Ver 3.0 Nov 2002	8	No data	0
hluc+ver2A4	Ver 3.0 Nov 2002	No data	No data	No data
hluc+ver2A5	Ver 3.0 Nov 2002	No data	No data	No data
hluc+ver2A6	Ver 3.0 Nov 2002	2	0	0
hluc+ver2A6	Ver 3.1.1 Apr 2003	4	0	0
hluc+ver2A7	Ver 3.1.1 Apr 2003	1	0	0
hluc+ver2A8	Ver 3.1.1 Apr 2003	1	0	0
hluc+ver2B1	Ver 3.0 Nov 2002	187	2	8
hluc+ver2B2	Ver 3.0 Nov 2002	No data	No data	No data
hluc+ver2B3	Ver 3.0 Nov 2002	35	No data	0
hluc+ver2B4	Ver 3.0 Nov 2002	No data	No data	No data
hluc+ver2B5	Ver 3.0 Nov 2002	No data	No data	No data
hluc+ver2B6	Ver 3.0 Nov 2002	2	0	0
hluc+ver2B6	Ver 3.1.1 Apr 2003	6	0	0
hluc+ver2B7	Ver 3.1.1 Apr 2003	2	0	0
hluc+ver2B8	Ver 3.1.1 Apr 2003	1	0	0
hluc+ver2B9	Ver 3.1.1 Apr 2003	1	0	0
hluc+ver2B10	Ver 3.1.1 Apr 2003	1	0	0
---	---	---	---	---
MCS-1	Ver 2.2 Sep 2001	14	No data	(not applicable)

Sequence	Matrix Library	TFBS (family matches)	Promoter modules	Splice sites (+ strand)
MCS-2	Ver 2.2 Sep 2001	12	No data	(not applicable)
MCS-3	Ver 2.2 Sep 2001	0	No data	(not applicable)
MCS-4	Ver 2.3 Feb 2001	0	0	(not applicable)
---	---	---	---	---
Bla	(not applicable)	No data	No data	(not applicable)
bla-1	Ver 2.2 Sep 2001	94	1	(not applicable)
bla-2	Ver 2.3 Feb 2001	51	No data	(not applicable)
bla-3	Ver 2.3 Feb 2001	16	No data	(not applicable)
bla-4	Ver 2.3 Feb 2001	14	No data	(not applicable)
bla-5	Ver 2.3 Feb 2001	5	0	(not applicable)
---	---	---	---	---
pGL4B-4NN	Ver 2.4 May 2002	11	0	(not applicable)
pGL4B-4NN1	Ver 2.4 May 2002	7	No data	(not applicable)
pGL4B-4NN2	Ver 2.4 May 2002	4	0	(not applicable)
pGL4B-4NN3	Ver 2.4 May 2002	3	0	(not applicable)
---	---	---	---	---
*Spe*I-*Nco*I-Ver2-Start	Ver 4.0 Nov 2003	34	1	(not applicable)
*Spe*I-*Nco*I-Ver2	Ver 4.0 Nov 2003	28	1	(not applicable)

Using the 5 sequences, i.e., hluc+ver2A1, bla-1, hneo-1, hpuro-1, hhyg-1 (humanized codon usage) for analysis, TFBS from the following families were found in 3 out 5 sequences:

V$AHRR (AHR-arnt heterodimers and AHR-related factors)

V$ETSF (Human and murine ETS1 factors)

V&NFKB (Nuclear Factor Kappa B/c-rel)

V$VMYB (AMV-viral myb oncogene)

V$CDEF (Cell cycle regulators: Cell cycle dependent element)

V$HAND (bHLH transcription factor dimer of HAND2 and E12)

V$NRSF (Neuron-Restrictive Silencer Factor)

V$WHZF (Winged Helix and ZF5 binding sites)

V$CMYB (C-myb, cellular transcriptional activator)

V$MINI (Muscle INItiator)

V$P53F (p53 tumor suppr.-neg. regulat. of the tumor suppr. Rb)

V$ZF5F (ZF5 POZ domain zinc finger)

V$DEAF (Homolog to deformed epidermal autoregulatory factor-1 from *D. melanogaster*)

V$MYOD (MYOblast Determining factor)

V$PAX5 (PAX-5/PAX-9 B-cell-specific activating protein)

V$EGRF (EGR/nerve growth Factor Induced protein C & rel. fact.)

V$NEUR (NeuroD, Beta2, HLH domain)

V$REBV (Epstein-Barr virus transcription factor R);

TFBS from the following families were found in 4 out of 5 sequences:

V$ETSF (Human and murine ETS1 factors)

V$CDEF (Cell cycle regulators: Cell cycle dependent element)

V$HAND (bHLH transcription factor dimer of HAND2 and E12)

V$NRSF (Neuron-Restrictive Silencer Factor)

V$PAX5 (PAX-5/PAX-9 B-cell-specific activating protein)

V$NEUR (NeuroD, Beta2, HLH domain); and

TFBS from the following families were found in 5 out of 5 sequences:

V$PAX5 (PAX-5/PAX-9 B-cell-specific activating protein).

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All publications, patents and patent applications are incorporated herein by reference. While in the foregoing specification, this invention has been described in relation to certain preferred embodiments thereof, and many details have been set forth for purposes of illustration, it will be apparent to those skilled in the art that the invention is susceptible to additional embodiments and that certain of the details herein may be varied considerably without departing from the basic principles of the invention.

WHAT IS CLAIMED IS:

1. An isolated nucleic acid molecule comprising a synthetic nucleotide sequence having a coding region for a selectable polypeptide, wherein the synthetic nucleotide sequence has 90% or less nucleic acid sequence identity to a parent nucleic acid sequence encoding a corresponding selectable polypeptide, wherein the decreased sequence identity is a result of different codons in the synthetic nucleotide sequence relative to the codons in the parent nucleic acid sequence, wherein the nucleotide sequence encodes a selectable polypeptide with at least 85% amino acid sequence identity to the corresponding selectable polypeptide encoded by the parent nucleic acid sequence, wherein the synthetic nucleotide sequence has a reduced number of regulatory sequences relative to the average number of regulatory sequences resulting from random selections of codons at the sequences which differ between the synthetic nucleotide sequence and the parent nucleic acid sequence, and wherein the synthetic nucleotide sequence, when expressed in a cell, confers resistance to ampicillin, puromycin, hygromycin or neomycin.

2. The isolated nucleic acid molecule of claim 1 wherein the regulatory sequences include transcription factor binding sequences, intron splice sites, poly(A) sites, promoter modules, and/or promoter sequences.

3. The isolated nucleic acid molecule of claim 1 wherein a majority of the codons which differ are ones that are preferred codons of a desired host cell and/or are not low-usage codons in that host cell.

4. The isolated nucleic acid molecule of claim 3 wherein the majority of the codons which differ in the synthetic nucleic acid sequence are those which are employed more frequently in mammals.

5. The isolated nucleic acid molecule of claim 3 wherein the majority of the codons which differ in the synthetic nucleic acid sequence are those which are preferred codons in humans.

6. The isolated nucleic acid molecule of claim 3 wherein the majority of codons which differ are the codons CGC, CTG, AGC, ACC, CCC, GCC, GGC, GTG, ATC, AAG, AAC, CAG, CAC, GAG, GAC, TAC, TGC and TTC.

7. The isolated nucleic acid molecule of claim 1 wherein the nucleic acid molecule encodes a fusion of the selectable polypeptide with a luciferase.

8. The isolated nucleic acid molecule of claim 7 wherein the luciferase is a *Renilla* luciferase, a firefly luciferase or a click beetle luciferase.

9. The isolated nucleic acid molecule of claim 1 wherein the parent nucleic acid sequence is a wild-type *neo*, *hyg*, *bla* or *puro* sequence.

10. The isolated nucleic acid molecule of claim 1 wherein the parent nucleic acid sequence is SEQ ID NO:1, SEQ ID NO:6, SEQ ID NO:15 or SEQ ID NO:41.

11. The isolated nucleic acid molecule of claim 1 wherein the synthetic nucleotide sequence comprises an open reading frame in SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:30, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:42, SEQ ID NO:44; SEQ ID NO:70, SEQ ID NO:71, SEQ ID NO:72, SEQ ID NO:73, SEQ ID NO:74, SEQ ID NO:80, SEQ ID NO:81, SEQ ID NO:82, SEQ ID NO:83, or SEQ ID NO:84.

12. The isolated nucleic acid molecule of claim 1 wherein the synthetic nucleotide sequence has at least 10% fewer regulatory sequences.

13. The isolated nucleic acid molecule of claim 1 wherein the synthetic nucleotide sequence has an increased number of AGC serine-encoding codons, an increased number of ATC isoleucine-encoding codons, an increased number of CCC proline-encoding codons, and/or an increased number of ACC threonine-encoding codons.

14. The isolated nucleic acid molecule of claim 1 wherein the codons in the synthetic nucleotide sequence which differ encode the same amino acids as the corresponding codons in the parent nucleic acid sequence.

15. The isolated nucleic acid molecule of claim 1 which has at least 90% nucleotide sequence identity to an open reading frame in any one of SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:30, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:42, SEQ ID NO:44, SEQ ID NO:70, SEQ ID NO:71, SEQ ID NO:72, SEQ ID NO:73, SEQ ID NO:74, SEQ ID NO:80, SEQ ID NO:81, SEQ ID NO:82, SEQ ID NO:83, or SEQ ID NO:84, or the complement thereof.

16. The isolated nucleic acid molecule of claim 1 wherein the nucleic acid molecule encodes a fusion of the selectable polypeptide with one or more other peptides or polypeptides, wherein at least the selectable polypeptide is encoded by the synthetic nucleic acid sequence.

17. The isolated nucleic acid molecule of claim 16 wherein one or more other peptides are peptides having protein destabilization sequences.

18. A plasmid comprising the nucleic acid molecule of claim 1.

19. The plasmid of claim 18 which further comprises a multiple cloning region.

20. The plasmid of claim 18 which further comprises an open reading frame of interest.

21. The plasmid of claim 18 which further comprises a promoter functional in a particular host cell operably linked to the synthetic nucleotide sequence.

22. The plasmid of claim 21 wherein the promoter is functional in a prokaryotic cell.

23. The plasmid of claim 21 wherein the promoter is functional in a eukaryotic cell.

24. The plasmid of claim 20 further comprising a promoter operably linked to the open reading frame of interest.

25. An isolated nucleic acid molecule comprising a synthetic nucleotide sequence encoding a firefly luciferase, wherein the synthetic nucleotide sequence has 80% or less nucleic acid sequence identity to a parent nucleic acid sequence having SEQ ID NO:43 or 85% or less nucleic acid sequence identity to a parent nucleic acid sequence having SEQ ID NO:14 which encodes a firefly luciferase, wherein the decreased sequence identity is a result of different codons in the synthetic nucleotide sequence relative to the codons in the parent nucleic acid sequence, wherein the synthetic nucleotide sequence encodes a firefly luciferase which has at least 85% amino acid sequence identity to the corresponding luciferase encoded by the parent nucleic acid sequence, and wherein the synthetic nucleotide sequence has a reduced number of regulatory sequences relative to the average number of regulatory sequences resulting from random selections of codons at the sequences which differ between the synthetic nucleotide sequence and the parent nucleic acid sequence.

26. The isolated nucleic acid molecule of claim 25 wherein the regulatory sequences include transcription factor binding sequences, intron splice

sites, poly(A) sites, promoter modules, and/or promoter sequences.

27. The isolated nucleic acid molecule of claim 25 wherein a majority of the codons which differ are ones that are preferred codons of a desired host cell and/or are not low-usage codons in that host cell.

28. The isolated nucleic acid molecule of claim 27 wherein the majority of the codons which differ in the synthetic nucleic acid molecule are those which are employed more frequently in mammals.

29. The isolated nucleic acid molecule of claim 27 wherein the majority of the codons which differ in the synthetic nucleic acid molecule are those which are preferred codons in humans.

30. The isolated nucleic acid molecule of claim 27 wherein the majority of codons which differ are the codons CGC, CTG, AGC, ACC, CCC, GCC, GGC, GTG, ATC, AAG, AAC, CAG, CAC, GAG, GAC, TAC, TGC and TTC.

31. The isolated nucleic acid molecule of claim 25 wherein the synthetic nucleotide sequence comprises a sequence in an open reading frame in SEQ ID NO:21, SEQ ID NO:22, or SEQ ID NO:23 or has at least 90% nucleotide sequence identity thereto.

32. The isolated nucleic acid molecule of claim 25 wherein the synthetic nucleic acid molecule is expressed in a mammalian host cell at a level which is greater than that of the parent nucleic acid sequence.

33. The isolated nucleic acid molecule of claim 25 wherein the synthetic nucleic acid molecule has an increased number of AGC serine-encoding codons, an increased number of CCC proline-encoding codons, an increased number of ATC isoleucine-encoding codons and/or an increased number of ACC threonine-encoding codons.

34. The isolated acid molecule of claim 25 wherein the synthetic nucleotide sequence has at least 10% fewer transcription regulatory sequences .

35. The isolated nucleic acid molecule of claim 25 wherein the codons in the synthetic nucleotide sequence which differ encode the same amino acids as the corresponding codons in the parent nucleic acid sequence.

36. The isolated nucleic acid molecule of claim 25 wherein the nucleic acid molecule encodes a fusion of the luciferase with one or more other peptides or polypeptides, wherein at least the luciferase is encoded by the synthetic nucleic acid sequence.

37. The isolated nucleic acid molecule of claim 36 wherein one or more other peptides are peptides having protein destabilization sequences.

38. A plasmid comprising the nucleic acid molecule of claim 25.

39. The plasmid of claim 38 which further comprises a multiple cloning region.

40. The plasmid of claim 38 which further comprises a promoter operatively linked to the synthetic nucleotide sequence.

41. The plasmid of claim 38 which further comprises the synthetic nucleotide sequence of the nucleic acid molecule of claim 1.

42. An expression vector comprising the nucleic acid molecule of claim 25 linked to a promoter functional in a cell.

43. The expression vector of claim 42 wherein the promoter is functional in a eukaryotic cell.

44. The expression vector of claim 42 wherein the expression vector further comprises a multiple cloning site.

45. The expression vector of claim 42 wherein the promoter is functional in a mammalian cell.

46. The expression vector of claim 42 wherein the synthetic nucleotide sequence is operatively linked to a Kozak consensus sequence.

47. A plasmid comprising a nucleotide sequence comprising SEQ ID NO:74 or a nucleotide sequence comprising at least 80% nucleic acid sequence identity to SEQ ID NO:74, which nucleotide sequence comprises an open reading frame with less than 90% nucleic acid sequence identity to SEQ ID NO:41, and the expression of which open reading frame in a host cell confers resistance to ampicillin.

48. A host cell comprising the expression cassette of claim 42.

49. A host cell comprising the plasmid of claim 17, 38 or 47.

50. A kit comprising, in suitable container means, the plasmid of claim 17, 38 or 47.

51. A polynucleotide which hybridizes under stringent hybridization conditions to SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:30, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:42, SEQ ID NO:44, SEQ ID NO:70, SEQ ID NO:71, SEQ ID NO:72, SEQ ID NO:73, SEQ ID NO:74, SEQ ID NO:80, SEQ ID NO:81, SEQ ID NO:82, SEQ ID NO:83, SEQ ID NO:84, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, or the complement of the polynucleotide, wherein the polynucleotide or the complement thereof encodes a selectable polypeptide or a firefly luciferase.

52. The polynucleotide of claim 51 which does not have SEQ ID NO:1, SEQ ID NO:6, SEQ ID NO:15, SEQ ID NO:41, SEQ ID NO:14, or SEQ ID NO:43.

53. An isolated nucleic acid molecule comprising a synthetic nucleotide sequence which does not code for a desirable peptide or polypeptide but includes sequences which inhibit transcription and/or translation, wherein the synthetic nucleotide sequence has at least 20 nucleotides which have a different sequence relative to a corresponding parent nucleic acid sequence which does not code for the desirable peptide or polypeptide, wherein the synthetic nucleotide sequences has 90% or less nucleic acid sequence identity to the parent nucleic acid sequences, and wherein the sequence difference is a result of a reduced number of one or more regulatory sequences in the synthetic nucleotide sequence relative to the parent nucleic acid sequence.

54. The isolated nucleic acid molecule of claim 53 wherein the synthetic nucleotide sequence has SEQ ID NO:49.

55. The isolated nucleic acid molecule of claim 53 further comprising a multiple cloning region and/or a poly(A) site.

56. The isolated nucleic acid molecule of claim 53 wherein the sequences which inhibit transcription include one or more poly(A) sites.

57. The isolated nucleic acid molecule of claim 53 wherein the sequences which inhibit translation include one or more stop codons in one or more reading frames.

58. The isolated nucleic acid molecule of claim 53 wherein the parent nucleic acid sequence includes a multiple cloning region.

59. The isolated nucleic acid molecule of claim 53 wherein the parent nucleic acid sequence includes sequences which inhibit transcription and/or translation.

60. The isolated nucleic acid molecule of claim 53 wherein the parent nucleic acid sequence has SEQ ID NO:76.

61. The isolated nucleic acid molecule of claim 53 wherein the synthetic nucleotide sequence has a reduced number of one or more restriction endonuclease recognition sites relative to the parent nucleic acid sequence.

62. A plasmid comprising the nucleic acid molecule of claim 53.

63. A plasmid which includes a sequence including SEQ ID NO:89, SEQ ID NO:90, or a sequence having at least 90% nucleic acid sequence identity thereto, or the complement thereof, which sequence encodes at least one selectable and/or screenable polypeptide.

64. The plasmid of claim 63 further comprising a multiple cloning region.

65. The plasmid of claim 63 further comprising another selectable or screenable polypeptide.

66. The plasmid of claim 63 or 65 wherein at least one selectable or screenable polypeptide comprises one or more protein destabilization sequences.

67. The plasmid of claim 63 wherein the sequence for the at least one selectable and/or screenable polypeptide is not SEQ ID NO:41.

68. A synthetic nucleotide sequence of at least 100 nucleotides having a coding region for a selectable polypeptide which confers resistance to ampicillin, puromycin, hygromycin or neomycin, wherein the synthetic

nucleotide sequence has 90% or less nucleic acid sequence identity to a corresponding region of a parent nucleic acid sequence for the selectable polypeptide, wherein the decreased sequence identity is a result of different codons in the synthetic nucleotide sequence relative to the codons in the corresponding region in the parent nucleic acid sequence, wherein the synthetic nucleotide sequence has a reduced number of regulatory sequences relative to the average number of regulatory sequences resulting from random selections of codons at the sequences which differ between the synthetic nucleotide sequence and the parent nucleic acid sequence.

69. An isolated nucleic acid molecule encoding a selectable polypeptide and comprising a synthetic nucleotide sequence of at least 100 nucleotides having a coding region for the selectable polypeptide, wherein the synthetic nucleotide sequence has 90% or less nucleic acid sequence identity to a corresponding region in a parent nucleic acid sequence for the selectable polypeptide, wherein the decreased sequence identity is a result of different codons in the synthetic nucleotide sequence relative to the codons in the parent nucleic acid sequence, wherein the synthetic nucleotide sequence encodes a region of the selectable polypeptide with at least 85% amino acid sequence identity to the corresponding region of the selectable polypeptide encoded by the parent nucleic acid sequence, wherein the synthetic nucleotide sequence has a reduced number of regulatory sequences relative to the average number of regulatory sequences resulting from random selections of codons at the sequences which differ between the synthetic nucleotide sequence and the parent nucleic acid sequence, and wherein the isolated nucleic acid molecule, when expressed in a cell, confers resistance to ampicillin, puromycin, hygromycin or neomycin.

Figure 1

Amino Acid	Codon
Phe	UUU, UUC
Ser	UCU, UCC, UCA, UCG, AGU, AGC
Tyr	UAU, UAC
Cys	UGU, UGC
Leu	UUA, UUG, CUU, CUC, CUA, CUG
Trp	UGG
Pro	CCU, CCC, CCA, CCG
His	CAU, CAC
Arg	CGU, CGC, CGA, CGG, AGA, AGG
Gln	CAA, CAG
Ile	AUU, AUC, AUA
Thr	ACU, ACC, ACA, ACG
Asn	AAU, AAC
Lys	AAA, AAG
Met	AUG
Val	GUU, GUC, GUA, GUG
Ala	GCU, GCC, GCA, GCG
Asp	GAU, GAC
Gly	GGU, GGC, GGA, GGG
Glu	GAA, GAG

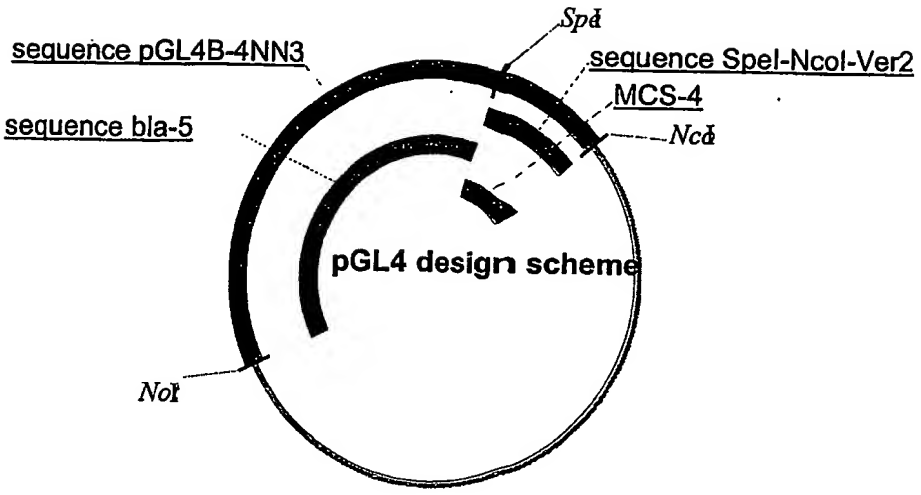
sequence pGL4B-4NN3
sequence bla-5
SpeI
sequence SpeI-NcoI-Ver2
MCS-4
NcoI
pGL4 design scheme
NotI

FIG. 2

SEQUENCE LISTING

<110> Promega Corporation
Wood, Keith
Wood, Monika
Almond, Brian
Paguio, Aileen
Fan, Frank

<120> Synthetic nucleic acid molecule and methods of preparation

<130> 341.034WO1

<160> 97

<170> FastSEQ for Windows Version 4.0

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<220>
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35 40 45
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50 55 60
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145 150 155 160
Glu His Gln Gly Leu Ala Pro Ala Glu Leu Phe Ala Arg Leu Lys Ala
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180 185 190
Leu Pro Asn Ile Met Val Glu Asn Gly Arg Phe Ser Gly Phe Ile Asp
195 200 205
Cys Gly Arg Leu Gly Val Ala Asp Arg Tyr Gln Asp Ile Ala Leu Ala
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Thr Arg Asp Ile Ala Glu Glu Leu Gly Gly Glu Trp Ala Asp Arg Phe
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35 40 45
Arg Val Asn Ser Cys Ala Asp Gly Phe Tyr Lys Asp Arg Tyr Val Tyr
50 55 60
Arg His Phe Ala Ser Ala Ala Leu Pro Ile Pro Glu Val Leu Asp Ile
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Thr Thr Trp Arg Asp Phe Ile Cys Ala Ile Ala Asp Pro His Val Tyr
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His Trp Gln Thr Val Met Asp Asp Thr Val Ser Ala Ser Val Ala Gln
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180 185 190

His Leu Val His Ala Asp Phe Gly Ser Asn Asn Val Leu Thr Asp Asn
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Gly Arg Ile Thr Ala Val Ile Asp Trp Ser Glu Ala Met Phe Gly Asp
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245 250 255
Ala Gly Ser Pro Arg Leu Arg Ala Tyr Met Leu Arg Ile Gly Leu Asp
260 265 270
Gln Leu Tyr Gln Ser Leu Val Asp Gly Asn Phe Asp Asp Ala Ala Trp
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ggcgaatggg ctgaccgctt cctcgtgctt tacggtatcg ccgctcccga ttcgcagcgc 1740
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<210> 13
<211> 1776
<212> DNA
<213> Artificial Sequence

<220>
<223> A synthetic construct.

<400> 13
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agcttcgtgg agcgcgtgct gaagaacgag cagtaa 1776

<210> 14
<211> 1653
<212> DNA
<213> Artificial Sequence

<220>
<223> A synthetic construct.

<400> 14
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aagaagctgc ctatcatcca gaagatcatc atcatggact ctaagaccga ctaccagggc 480
ttccagagca tgtacacatt cgtgacatct catctgcctc ctggcttcaa cgagtacgac 540
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gaggtgccca agggcctgac cggcaagctg gacgcccgca agatccgcga gatcctgatc 1620
aaggctaaga aaggcggcaa gatcgccgtg taa 1653

<210> 15
<211> 597
<212> DNA
<213> Streptomyces sp.

<400> 15
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cgcaccctcg ccgccgcgtt cgccgactac cccgccacgc gccacaccgt cgacccggac 120
cgccacatcg agcgggtcac cgagctgcaa gaactcttcc tcacgcgcgt cgggctcgac 180
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<210> 16
<211> 1672
<212> DNA
<213> Artificial Sequence

<220>
<223> A synthetic construct.

<400> 16
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gaggacggca ccgccggcga gcagctgcac aaggccatga agcgctacgc cctggtgccc 120
ggcaccatcg ccttcaccga cgcccacatc gaggtggaca tcacctacgc cgagtacttc 180
gagatgagcg tgcgcctggc cgaggccatg aagcgctacg gcctgaacac caaccaccgc 240
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ttcgtggacg aggtgcccaa gggcctgacc ggcaagctgg acgcccgcaa gatccgcgag 1620
atcctgatca aggccaagaa gggcggcaag atcgccgtgt aataattcta ga 1672

<210> 17
<211> 1672
<212> DNA
<213> Artificial Sequence

<220>
<223> A synthetic construct.

<400> 17
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gaggacggca ccgccggcga gcagctgcac aaggccatga agcgctacgc cctggtgccc 120
ggcaccatcg ccttcaccga cgcacatatc gaggtggaca tcacctacgc cgagtacttc 180
gagatgagcg ttcggctggc agaggctatg aagcgctatg ggctgaacac caaccatcgc 240
atcgtggtgt gcagcgagaa cagcttgcag ttcttcatgc ccgtgttggg tgccctgttc 300
atcggcgtgg ctgtggcccc agctaacgac atctacaacg agcgcgagct gctgaacagc 360
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ttcgtggacg aggtgcctaa aggcctgacg ggcaagttgg acgcccgcaa gatccgcgag 1620

attctgatca aggccaagaa gggcggcaag atcgccgtgt aataattcta ga 1672

<210> 18
<211> 1672
<212> DNA
<213> Artificial Sequence

<220>
<223> A synthetic construct.

<400> 18
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gaggacggca ccgccggcga gcagctgcac aaagccatga agcgctacgc cctggtgccc 120
ggcaccatcg cctttaccga cgcacatatc gaggtggaca tcacctacgc cgagtacttc 180
gagatgagcg ttcggctggc agaggctatg aagcgctatg ggctgaatac caaccatcgc 240
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atcggtgtgg ctgtggcccc agctaacgac atctacaacg agcgcgagct gctgaacagc 360
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ttcgtggacg aggtgcctaa aggcctgacg ggcaagttgg acgcccgcaa gatccgcgag 1620
attctcatta aggccaagaa gggcggcaag atcgccgtgt aataattcta ga 1672

<210> 19
<211> 1672
<212> DNA
<213> Artificial Sequence

<220>
<223> A synthetic construct.

<400> 19
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gaagacggca ccgccggcga gcagctgcac aaagccatga agcgctacgc cctggtgccc 120
ggcaccatcg cctttaccga cgcacatatc gaggtggaca ttacctacgc cgagtacttc 180
gagatgagcg ttcggctggc agaagctatg aagcgctatg ggctgaacac caaccatcgc 240
atcgtggtgt gcagcgagaa tagcttgcag ttcttcatgc ccgtgttggg tgccctgttc 300
atcggtgtgg ctgtggcccc agctaacgac atctacaacg agcgcgagct gctgaacagc 360
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ttcgtggacg aggtgcctaa aggcctgacg ggcaagttgg acgcccgcaa gatccgcgag 1620
attctcatta aggccaagaa gggcggcaag atcgccgtgt aataattcta ga 1672

<210> 20
<211> 1672
<212> DNA
<213> Artificial Sequence

<220>
<223> A synthetic construct.

<400> 20
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gaagacggca ccgccggcga gcagctgcac aaagccatga agcgctacgc cctggtgccc 120
ggcaccatcg cctttaccga cgcacatatc gaggtggaca ttacctacgc cgagtacttc 180
gagatgagcg ttcggctggc agaagctatg aagcgctatg ggctgaacac caaccatcgg 240
atcgtggtgt gcagcgagaa tagcttgcag ttcttcatgc ccgtgttggg tgccctgttc 300
atcggtgtgg ctgtggcccc agctaacgac atctacaacg agcgcgagct gctgaacagc 360
atgggcatca gccagcccac cgtcgtattc gtgagcaaga aagggctgca aaagatcctc 420
aacgtgcaaa agaagctacc gatcatacaa aagatcatca tcatggatag caagaccgac 480
taccagggct tccaaagcat gtacaccttc gtgacttccc atttgccacc cggcttcaac 540
gagtacgact tcgtgcccga gagcttcgac cgggacaaaa ccatcgccct gatcatgaac 600
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<210> 21
<211> 1672
<212> DNA
<213> Artificial Sequence

<220>
<223> A synthetic construct.

<400> 21
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aacgtgcaaa agaagctacc gatcatacaa aagatcatca tcatggatag caagaccgac 480
taccagggct tccaaagcat gtacaccttc gtgacttccc atttgccacc cggcttcaac 540
gagtacgact tcgtgcccga gagcttcgac cgggacaaaa ccatcgccct gatcatgaac 600
agtagtggca gtaccggatt gcccaagggc gtagccctac cgcaccgcac cgcttgtgtc 660
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<210> 22
<211> 1672
<212> DNA
<213> Artificial Sequence

<220>
<223> A synthetic construct.

<400> 22
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gagtacgact tcgtgcccga gagcttcgac cgggacaaaa ccatcgccct gatcatgaac 600
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ttcgtggacg aggtgcctaa aggcctgacg ggcaagttgg acgcccgcaa gatccgcgag 1620
attctcatta aggccaagaa gggcggcaag atcgccgtgt aataattcta ga 1672

<210> 23
<211> 1672
<212> DNA
<213> Artificial Sequence

<220>
<223> A synthetic construct.

<400> 23
aaagccacca tggaagatgc caaaaacatt aagaagggcc cagcgccatt ctacccactc 60
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ggcaccatcg cctttaccga cgcacatatc gaggtggaca ttacctacgc cgagtacttc 180
gagatgagcg ttcggctggc agaagctatg aagcgctatg ggctgaatac aaaccatcgg 240
atcgtggtgt gcagcgagaa tagcttgcag ttcttcatgc ccgtgttggg tgccctgttc 300
atcggtgtgg ctgtggcccc agctaacgac atctacaacg agcgcgagct gctgaacagc 360
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ttcgtggacg aggtgcctaa aggactgacc ggcaagttgg acgcccgcaa gatccgcgag 1620
attctcatta aggccaagaa gggcggcaag atcgccgtgt aataattcta ga 1672

<210> 24
<211> 1672
<212> DNA
<213> Artificial Sequence

<220>
<223> A synthetic construct.

<400> 24
aaagccacca tggaggatgc taagaatatt aagaaggggc ctgctccttt ttatcctctg 60
gaggatggga cagctgggga gcagctgcat aaggctatga agagatatgc tctggtgcct 120
gggacaattg cttttacaga tgctcatatt gaggtggata ttacatatgc tgagtatttt 180
gagatgtctg tgagactggc tgaggctatg aagagatatg ggctgaatac aaatcataga 240
attgtggtgt gttctgagaa ttctctgcag ttttttatgc ctgtgctggg ggctctgttt 300
attggggtgg ctgtggctcc tgctaatgat atttataatg agagagagct gctgaattct 360
atggggattt ctcagcctac agtggtgttt gtgtctaaga aggggctgca gaagattctg 420
aatgtgcaga agaagctgcc tattattcag aagattatta ttatggattc taagacagat 480
tatcaggggt ttcagtctat gtatacattt gtgacatctc atctgcctcc tgggtttaat 540
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attctgatta aggctaagaa gggggggaag attgctgtgt aataattcta ga 1672

<210> 25
<211> 1672
<212> DNA
<213> Artificial Sequence

<220>
<223> A synthetic construct.

<400> 25
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gaggatggga ctgccgggga gcagctgcat aaagctatga agcggtatgc tctggtgcca 120
ggcacaattg cgttcacgga tgctcacatt gaggtggaca ttacatacgc tgagtatttt 180
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atcggggtgg ctgtggctcc tgctaacgac atttacaatg agagagagct tttgaactcg 360
atggggattt ctcagcctac agtggtgttt gtgagtaaga aagggcttca aaagattctc 420
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attctgatta aggctaagaa gggtggaaag attgctgtgt aatagttcta ga 1672

<210> 26
<211> 1672
<212> DNA
<213> Artificial Sequence

<220>
<223> A synthetic construct.

<400> 26
aaagccacca tggaagatgc taaaaacatt aagaaggggc ctgctccttt ctaccctctt 60
gaagatggga ctgctggcga gcaacttcac aaagctatga agcggtatgc tcttgtgcca 120
ggcacaattg cgttcacgga tgctcacatt gaggtggaca tcacatacgc tgagtatttt 180
gagatgtcgg tgcggctggc agaagctatg aagcgctatg ggctgaatac aaaccataga 240
attgtagtgt gcagtgagaa ctcgttgcag ttctttatgc ccgtgctggg ggctctcttc 300
atcggggtgg ctgtggctcc tgctaacgac atctacaacg agcgagagct gttgaactcg 360
atggggattt ctcagcctac agtggtgttt gtgagtaaga aagggcttca aaagattctc 420
aatgtgcaaa agaagctgcc tattattcaa aagattatta ttatggactc taagaccgac 480
taccaggggt ttcagtctat gtatacattt gtgacatctc atctgcctcc tggcttcaac 540
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<210> 27
<211> 1672
<212> DNA
<213> Artificial Sequence

<220>
<223> A synthetic construct.

<400> 27
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gaagatggga ctgctggcga gcaacttcac aaagctatga agcggtatgc tcttgtgcca 120
ggcacaattg cgttcacgga tgctcacatt gaggtggaca tcacatacgc tgagtatttt 180
gagatgtcgg tgcggctggc agaagctatg aagcgctatg ggctgaatac aaaccataga 240
attgtagtgt gcagtgagaa ctcgttgcag ttctttatgc ccgtgctggg ggctctcttc 300
atcggggtgg ctgtggctcc tgctaacgac atctacaacg agcgagagct gttgaactcg 360
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aatgtgcaaa agaagctgcc tattattcaa aagattatta ttatggactc taagacagac 480
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attctcatta aggctaagaa gggtggaaag attgctgtgt aatagttcta ga 1672

<210> 28
<211> 1672
<212> DNA
<213> Artificial Sequence

<220>
<223> A synthetic construct.

<400> 28
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gaagatggga ctgctggcga gcaacttcac aaagctatga agcggtatgc tcttgtgcca 120
ggcacaattg cgttcacgga tgctcacatt gaggtggaca tcacatacgc tgagtatttt 180
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attgtagtgt gcagtgagaa ctcgttgcag ttctttatgc ccgtgctggg ggctctcttc 300
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aatgtgcaaa agaagctgcc tattatacaa aagattatta ttatggactc taagaccgac 480
taccaggggt ttcagtccat gtacacattt gtaacctctc atctgcctcc tggcttcaac 540
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attctcatta aggctaagaa gggtggaaag attgctgtgt aatagttcta ga 1672

<210> 29
<211> 1672
<212> DNA
<213> Artificial Sequence

<220>
<223> A synthetic construct.

<400> 29
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gagatgtcgg tgcggctggc agaagctatg aagcgctatg ggctgaatac aaaccataga 240
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tttgtggatg aggtgcctaa agggctcact ggcaagctgg atgccagaaa aattcgagag 1620
attctcatta aggctaagaa gggtggaaag attgctgtgt aatagttcta ga 1672

<210> 30
<211> 1056
<212> DNA
<213> Artificial Sequence

<220>
<223> A synthetic construct.

<400> 30
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cgccgaggct atggacgcta ttgccgccgc cgacctgagc cagaccagcg gcttcggccc 420
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acgaccgcgc gctaaggagt agtaaccagc tcttgg 1056

<210> 31
<211> 1672
<212> DNA
<213> Artificial Sequence

<220>
<223> A synthetic construct.

<400> 31
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atcggggtgg ctgtggctcc tgctaacgac atctacaacg agcgagagct gttgaactcg 360
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aatgtgcaaa agaagctacc gatcatacaa aagatcatca tcatggatag caagaccgac 480
taccaggggt ttcagtccat gtacacattt gtaacctctc atctgcctcc tggcttcaac 540
gagtacgact tcgtgcccga gtctttcgac agggacaaaa cgattgctct gatcatgaac 600
agctccgggt ctaccgggct gcctaagggt gtagctctgc cccatcgaac agcttgtgtg 660
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<210> 32
<211> 1672
<212> DNA
<213> Artificial Sequence

<220>
<223> A synthetic construct.

<400> 32
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gagatgtcgg tgcggctggc agaagctatg aagcgctatg ggctgaatac aaaccataga 240
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attggggtgg ctgtggctcc tgctaatgac atctacaacg agcgagagct gttgaacagt 360
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aatgtgcaaa agaagctacc gatcatacaa aagatcatca tcatggatag caagaccgac 480
taccaggggt ttcagtccat gtacacattt gtaacctctc atctgcctcc tggcttcaat 540
gagtatgact tcgtgcccga gtctttcgac agggacaaaa cgattgctct gatcatgaac 600
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tttgtggatg aggtgcctaa agggctcact ggcaagctgg atgccagaaa aattcgagag 1620
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<210> 33
<211> 1672
<212> DNA
<213> Artificial Sequence

<220>
<223> A synthetic construct.

<400> 33
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gagatgtcgg tgcggctggc agaagctatg aagcgctatg ggctgaatac aaaccataga 240
attgtagtgt gcagtgagaa ctcgttgcag ttctttatgc ccgtgctggg ggctctcttc 300
attggggtgg ctgtggctcc tgctaatgac atctacaacg agcgagagct gttgaacagt 360
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agcagtgggt ctaccgggct gcctaagggt gtagctctgc cccatcgaac agcttgtgtg 660
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tttgtggatg aggtgcctaa aggactcact ggcaagctgg atgccagaaa aattcgagag 1620
attctcatta aggctaagaa gggtggaaag attgctgtgt aatagttcta ga 1672

<210> 34
<211> 10
<212> DNA
<213> Artificial Sequence

<220>
<223> A synthetic construct.

<400> 34
gccaccatga 10

<210> 35
<211> 11
<212> DNA
<213> Artificial Sequence

<220>
<223> A synthetic construct.

<220>
<221> misc_feature
<222> 4, 5, 6, 7, 8
<223> n = A,T,C or G

<400> 35
ccannnnntg g 11

<210> 36
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> A synthetic construct.

<220>
<221> misc_feature
<222> 1, 2, 3, 4, 5, 9, 10, 11, 12, 13
<223> n = A,T,C or G

<400> 36
nnnnnccann nnntggccac catgg 25

<210> 37
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> A synthetic construct.

<220>
<221> misc_feature
<222> 10, 11, 12, 13, 14, 18, 19, 20
<223> n = A,T,C or G

<400> 37
taataaccan nnnntggnnn 20

<210> 38
<211> 825
<212> DNA
<213> Artificial Sequence

<220>
<223> A synthetic construct.

<400> 38
ccactcagtg gccaccatga tcgagcagga cggcctccat gctggcagtc ccgcagcctg 60
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gtgggccgac cgcttcctgg tcttgtacgg catcgcagct cccgacagcc agcgcatcgc 780
cttctaccgc ctgctggacg agttcttcta gtaaccaggc tctgg 825

<210> 39
<211> 825
<212> DNA
<213> Artificial Sequence

<220>
<223> A synthetic construct.

<400> 39
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cgtgttccgt ctaagcgctc aaggccggcc cgtgctgttc gtgaagaccg acctgagcgg 180
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<210> 40

<400> 40
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<210> 41
<211> 861
<212> DNA
<213> Escherichia coli

<400> 41
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5cgcggtatca ttgcagcact ggggccagat ggtaagccct cccgtatcgt agttatctac 780
acgacgggga gtcaggcaac tatggatgaa cgaaatagac agatcgctga gataggtgcc 840
tcactgatta agcattggta a 861

<210> 42
10<211> 1056
<212> DNA
<213> Artificial Sequence

<220>
15<223> A synthetic construct.

<400> 42
ccactccgtg gccaccatga agaagcccga gctgaccgct accagcgttg aaaaatttct 60
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20agccttcagc ttcgatgtcg gcggacgcgg ctatgtactg cgggtgaata gctgcgctga 180
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cgaagtgttg gacatcggcg agttcagcga gagcctgaca tactgcatca gtagacgcgc 300
ccaaggcgtt actctccaag acctccccga aacagagctg cctgctgtgt tacagcctgt 360
cgccgaagct atggatgcta ttgccgccgc cgacctcagt caaaccagcg gcttcggccc 420
25attcgggccc caaggcatcg gccagtacac aacctggcgg gatttcattt gcgccattgc 480
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tcaagccctg gacgaactga tgctgtgggc cgaagactgt cccgaggtgc gccacctcgt 600
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35acgaccgcgc gctaaggagt agtaaccagc tcttgg 1056

<210> 43
<211> 1653
<212> DNA
40<213> Artificial Sequence

<220>
<223> A synthetic construct.

<400> 43
atggaagacg ccaaaaacat aaagaaaggc ccggcgccat tctatccgct ggaagatgga 60
accgctggag agcaactgca taaggctatg aagagatacg ccctggttcc tggaacaatt 120
gcttttacag atgcacatat cgaggtggac atcacttacg ctgagtactt cgaaatgtcc 180
5gttcggttgg cagaagctat gaaacgatat gggctgaata caaatcacag aatcgtcgta 240
tgcagtgaaa actctcttca attctttatg ccggtgttgg gcgcgttatt tatcggagtt 300
gcagttgcgc ccgcgaacga catttataat gaacgtgaat tgctcaacag tatgggcatt 360
tcgcagccta ccgtggtgtt cgtttccaaa aaggggttgc aaaaaatttt gaacgtgcaa 420
aaaaagctcc caatcatcca aaaaattatt atcatggatt ctaaaacgga ttaccaggga 480
10tttcagtcga tgtacacgtt cgtcacatct catctacctc ccggttttaa tgaatacgat 540
tttgtgccag agtccttcga tagggacaag acaattgcac tgatcatgaa ctcctctgga 600
tctactggtc tgcctaaagg tgtcgctctg cctcatagaa ctgcctgcgt gagattctcg 660
catgccagag atcctatttt tggcaatcaa atcattccgg atactgcgat tttaagtgtt 720
gttccattcc atcacggttt tggaatgttt actacactcg gatatttgat atgtggattt 780
15cgagtcgtct taatgtatag atttgaagaa gagctgtttc tgaggagcct tcaggattac 840
aagattcaaa gtgcgctgct ggtgccaacc ctattctcct tcttcgccaa aagcactctg 900
attgacaaat acgatttatc taatttacac gaaattgctt ctggtggcgc tcccctctct 960
aaggaagtcg gggaagcggt tgccaagagg ttccatctgc caggtatcag gcaaggatat 1020
gggctcactg agactacatc agctattctg attacacccg agggggatga taaaccgggc 1080
20gcggtcggta aagttgttcc atttttgaa gcgaaggttg tggatctgga taccgggaaa 1140
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tatgtaaaca atccggaagc gaccaacgcc ttgattgaca aggatggatg gctacattct 1260
ggagacatag cttactggga cgaagacgaa cacttcttca tcgttgaccg cctgaagtct 1320
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25caccccaaca tcttcgacgc aggtgtcgca ggtcttcccg acgatgacgc cggtgaactt 1440
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tacgtcgcca gtcaagtaac aaccgcgaaa aagttgcgcg gaggagttgt gtttgtggac 1560
gaagtaccga aaggtcttac cggaaaactc gacgcaagaa aaatcagaga gatcctcata 1620
aaggccaaga agggcggaaa gatcgccgtg taa 1653
30

<210> 44
<211> 1369
<212> DNA
35<213> Artificial Sequence

<220>
<223> A synthetic construct.

40<400> 44
ggatccgttt gcgtattggg cgctcttccg ctgatctgcg cagcaccatg gcctgaaata 60
acctctgaaa gaggaacttg gttagctacc ttctgaggcg gaaagaacca gctgtggaat 120
gtgtgtcagt tagggtgtgg aaagtcccca ggctccccag caggcagaag tatgcaaagc 180

atgcatctca attagtcagc aaccaggtgt ggaaagtccc caggctcccc agcaggcaga 240
agtatgcaaa gcatgcatct caattagtca gcaaccatag tcccgcccct aactccgccc 300
atcccgcccc taactccgcc cagttccgcc cattctccgc cccatggctg actaattttt 360
tttatttatg cagaggccga ggccgcctct gcctctgagc tattccagaa gtagtgagga 420
5ggctttttg gaggcctagg cttttgcaaa aagctcgatt cttctgacac tagcgccacc 480
atgatcgaac aagacggcct ccatgctggc agtcccgcag cttgggtcga acgcttgttc 540
gggtacgact gggcccagca gaccatcgga tgtagcgatg cggccgtgtt ccgtctaagc 600
gctcaaggcc ggcccgtgct gttcgtgaag accgacctga gcggcgccct gaacgagctt 660
caagacgagg ctgcccgcct gagctggctg gccaccaccg gcgtaccctg cgccgctgtg 720
10ttggatgttg tgaccgaagc cggccgggac tggctgctgc tgggcgaggt ccctggccag 780
gatctgctga gcagccacct tgcccccgct gagaaggttt ctatcatggc cgatgcaatg 840
cggcgcctgc acaccctgga ccccgctacc tgcccccttcg accaccaggc taagcatcgg 900
atcgagcgtg ctcggacccg catggaggcc ggcctggtgg accaggacga cctggacgag 960
gagcatcagg gcctggcccc cgctgaactg ttcgcccgac tgaaagcccg catgccggac 1020
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ttagtcttgt acggcatcgc agctcccgac agccagcgca tcgccttcta ccgcttgctc 1260
gacgagttct tttaatgatc tagaaccggt catggccgca ataaaatatc tttattttca 1320
20ttacatctgt gtgttggttt tttgtgtgtt cgaactagat gctgtcgac 1369

<210> 45
<211> 1214
<212> DNA
25<213> Artificial Sequence

<220>
<223> A synthetic construct.

30<400> 45
gcggccgcaa atgctaaacc actgcagtgg ttaccagtgc ttgatcagtg aggcaccgat 60
ctcagcgatc tgcctatttc gttcgtccat agtggcctga ctccccgtcg tgtagatcac 120
tacgattcgt gagggcttac catcaggccc cagcgcagca atgatgccgc gagagccgcg 180
ttcaccggcc cccgatttgt cagcaatgaa ccagccagca gggagggccg agcgaagaag 240
35tggtcctgct actttgtccg cctccatcca gtctatgagc tgctgtcgtg atgctagagt 300
aagaagttcg ccagtgagta gtttccgaag agttgtggcc attgctactg gcatcgtggt 360
atcacgctcg tcgttcggta tggcttcgtt caactctggt tcccagcggt caagccgggt 420
cacatgatca cccatattat gaagaaatgc agtcagctcc ttagggcctc cgatcgttgt 480
cagaagtaag ttggccgcgg tgttgtcgct catggtaatg gcagcactac acaattctct 540
40taccgtcatg ccatccgtaa gatgcttttc cgtgaccggc gagtactcaa ccaagtcgtt 600
ttgtgagtag tgtatacggc gaccaagctg ctcttgcccg gcgtctatac gggacaacac 660
cgcgccacat agcagtactt tgaaagtgct catcatcggg aatcgttctt cggggcggaa 720
agactcaagg atcttgccgc tattgagatc cagttcgata tagcccactc ttgcacccag 780

ttgatcttca gcatctttta ctttcaccag cgtttcgggg tgtgcaaaaa caggcaagca 840
aaatgccgca aagaagggaa tgagtgcgac acgaaaatgt tggatgctca tactcttcct 900
ttttcaatat gtttgcagca tttgtcaggg ttactagtac gtctctcttg agagaccgcg 960
atcgccacca tgtctaggta ggtagtaaac gaaagggctt aaaggcctaa gtggccctcg 1020
agtccagcct tgagttggtt gagtccaagt cacgtttgga gatctggtac cttacgcgta 1080
tgagggttga gtccaagtca cgtttggaga tctggtacct tacgcgtatg agctctacgt 1140
agctagcggc ctcggcggcc gaattcttgc gttcgaagct tggcaatccg gtactgttgg 1200
taaagccacc atgg 1214

<210> 46
<211> 1522
<212> DNA
<213> Artificial Sequence

<220>
<223> A synthetic construct.

<400> 46
gcggccgcaa atgctaaacc actgcagtgg ttaccagtgc ttgatcagtg aggcaccgat 60
ctcagcgatc tgcctatttc gttcgtccat agtggcctga ctcccgtcg tgtagatcac 120
tacgattcgt gagggcttac catcaggccc cagcgcagca atgatgccgc gagagccgcg 180
ttcaccggcc cccgatttgt cagcaatgaa ccagccagca gggagggccg agcgaagaag 240
tggtcctgct actttgtccg cctccatcca gtctatgagc tgctgtcgtg atgctagagt 300
aagaagttcg ccagtgagta gtttccgaag agttgtggcc attgctactg gcatcgtggt 360
atcacgctcg tcgttcggta tggcttcgtt caactctggt tcccagcggt caagccgggt 420
cacatgatca cccatattat gaagaaatgc agtcagctcc ttagggcctc cgatcgttgt 480
cagaagtaag ttggccgcgg tgttgtcgct catggtaatg gcagcactac acaattctct 540
taccgtcatg ccatccgtaa gatgcttttc cgtgaccggc gagtactcaa ccaagtcgtt 600
ttgtgagtag tgtatacggc gaccaagctg ctcttgcccg gcgtctatac gggacaacac 660
cgcgccacat agcagtactt tgaaagtgct catcatcggg aatcgttctt cggggcggaa 720
agactcaagg atcttgccgc tattgagatc cagttcgata tagcccactc ttgcacccag 780
ttgatcttca gcatctttta ctttcaccag cgtttcgggg tgtgcaaaaa caggcaagca 840
aaatgccgca aagaagggaa tgagtgcgac acgaaaatgt tggatgctca tactcttcct 900
ttttcaatat gtttgcagca tttgtcaggg ttactagtac gtctctcaag agatttgtgc 960
atacacagtg actcatactt tcaccaatac tttgcatttt ggataaatac tagacaactt 1020
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ttgtaatgta ttttgtgtga tacccagagg tttaaggcaa cctattactc ttatgctcct 1140
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gcagtataat ttcagtgctt ttaaattttg tcctgcttac tattttcctt ttttatttgg 1260
gtttgatatg cgtgcacaga atggggcttc tattaaaata ttcttgagag accgcgatcg 1320
ccaccatgtc taggtaggta gtaaacgaaa gggcttaaag gcctaagtgg ccctcgagtc 1380
cagccttgag ttggttgagt ccaagtcacg tttggagatc tggtacctta cgcgtatgag 1440
ctctacgtag ctagcggcct cggcggccga attcttgcgt tcgaagcttg gcaatccggt 1500

actgttggta aagccaccat gg 1522

<210> 47
<211> 1134
<212> DNA
<213> Artificial Sequence

<220>
<223> A synthetic construct.

<400> 47
gcggccgcaa atgctaaacc actgcagtgg ttaccagtgc ttgatcagtg aggcaccgat 60
ctcagcgatc tgcctatttc gttcgtccat agtggcctga ctccccgtcg tgtagatcac 120
tacgattcgt gagggcttac catcaggccc cagcgcagca atgatgccgc gagagccgcg 180
ttcaccggcc cccgatttgt cagcaatgaa ccagccagca gggagggccg agcgaagaag 240
tggtcctgct actttgtccg cctccatcca gtctatgagc tgctgtcgtg atgctagagt 300
aagaagttcg ccagtgagta gtttccgaag agttgtggcc attgctactg gcatcgtggt 360
atcacgctcg tcgttcggta tggcttcgtt caactctggt tcccagcggt caagccgggt 420
cacatgatca cccatattat gaagaaatgc agtcagctcc ttagggcctc cgatcgttgt 480
cagaagtaag ttggccgcgg tgttgtcgct catggtaatg gcagcactac acaattctct 540
taccgtcatg ccatccgtaa gatgcttttc cgtgaccggc gagtactcaa ccaagtcgtt 600
ttgtgagtag tgtatacggc gaccaagctg ctcttgcccg gcgtctatac gggacaacac 660
cgcgccacat agcagtactt tgaaagtgct catcatcggg aatcgttctt cggggcggaa 720
agactcaagg atcttgccgc tattgagatc cagttcgata tagcccactc ttgcacccag 780
ttgatcttca gcatctttta ctttcaccag cgtttcgggg tgtgcaaaaa caggcaagca 840
aaatgccgca aagaagggaa tgagtgcgac acgaaaatgt tggatgctca tactcgtcct 900
ttttcaatat tattgaagca tttatcaggg ttactagtac gtctctcaag agatttgtgc 960
atacacagtg actcatactt tcaccaatac tttgcatttt ggataaatac tagacaactt 1020
tagaagtgaa ttatttatga ggttgtctta aaattaaaaa ttacaaagta ataaatcaca 1080
ttgtaatgta ttttgtgtga tacccagagg tttaaggcaa cctattactc ttat 1134

<210> 48
<211> 319
<212> DNA
<213> Artificial Sequence

<220>
<223> A synthetic construct.

<400> 48
actagtacgt ctctcaagga taagtaagta atattaaggt acgggaggta cttggagcgg 60
ccgcaataaa atatctttat tttcattaca tctgtgtgtt ggtttttttgt gtgaatcgat 120
agtactaaca tacgctctcc atcaaaacaa aacgaaacaa aacaaactag caaaataggc 180

tgtccccagt gcaagtgcag gtgccagaac atttctctgg cctaagtggc cggtaccgag 240
ctcgctagcc tcgaggatat cagatctggc ctcggcggcc aagcttggca atccggtact 300
gttggtaaag ccaccatgg 319

<210> 49
<211> 320
<212> DNA
<213> Artificial Sequence

<220>
<223> A synthetic construct.

<400> 49
actagtacgt ctctcaagga taagtaagta atattaaggt acgggaggta ttggacaggc 60
cgcaataaaa tatctttatt ttcattacat ctgtgtgttg gtttttgtgt tgaatcgata 120
gtactaacat acgctctcca tcaaaacaaa acgaaacaaa acaaactagc aaaataggct 180
gtccccagtg caagtgcagg tgccagaaca tttctctggc ctaactggcc ggtacctgag 240
ctcgctagcc tcgaggatat caagatctgg cctcggcggc caagcttggc aatccggtac 300
tgttggtaaa gccaccatgg 320

<210> 50
<211> 5
<212> DNA
<213> Artificial Sequence

<220>
<223> A synthetic construct.

<400> 50
tataa 5

<210> 51
<211> 6
<212> DNA
<213> Artificial Sequence

<220>
<223> A synthetic construct.

<400> 51
stratg 6

<210> 52
<211> 9
<212> DNA
<213> Artificial Sequence

<220>
<223> A synthetic construct.

<220>
<221> misc_feature
<222> 4, 6, 7
<223> n = A,T,C or G

<400> 52
mttncnnma 9

<210> 53
<211> 5
<212> DNA
<213> Artificial Sequence

<220>
<223> A synthetic construct.

<400> 53
tratg 5

<210> 54
<211> 38
<212> DNA
<213> Artificial Sequence

<220>
<223> A synthetic construct.

<400> 54
gtactgagac gacgccagcc caagcttagg cctgagtg 38

<210> 55
<211> 38
<212> DNA
<213> Artificial Sequence

<220>
<223> A synthetic construct.

<400> 55
5ggcatgagcg tgaactgact gaactagcgg ccgccgag 38

<210> 56
<211> 24
<212> DNA
10<213> Artificial Sequence

<220>
<223> A synthetic construct.

15<400> 56
ggatcccatg gtgaagcgtg agaa 24

<210> 57
<211> 21
20<212> DNA
<213> Artificial Sequence

<220>
<223> A synthetic construct.
25
<400> 57
ggatcccatg gtgaaacgcg a 21

<210> 58
30<211> 31
<212> DNA
<213> Artificial Sequence

<220>
35<223> A synthetic construct.

<400> 58
ctagcttttt tttctagata atcatgaaga c 31

40<210> 59
<211> 32
<212> DNA
<213> Artificial Sequence

<220>
<223> A synthetic construct.

<400> 59
5gcgtagccat ggtaaagcgt gagaaaaatg tc 32

<210> 60
<211> 33
<212> DNA
10<213> Artificial Sequence

<220>
<223> A synthetic construct.

15<400> 60
ccgactctag attactaacc gccggccttc acc 33

<210> 61
<211> 54
20<212> DNA
<213> Artificial Sequence

<220>
<223> A synthetic construct.
25
<400> 61
caaaaagctt ggcattccgg tactgttggt aaagccacca tggtgaagcg agag 54

<210> 62
30<211> 26
<212> DNA
<213> Artificial Sequence

<220>
35<223> A synthetic construct.

<400> 62
caattgttgt tgttaacttg tttatt 26

40<210> 63

<400> 63
000

<210> 64

<400> 64
000

<210> 65
<211> 10
<212> DNA
<213> Artificial Sequence

<220>
<223> A synthetic construct.

<400> 65
caccatggct 10

<210> 66
<211> 40
<212> DNA
<213> Artificial Sequence

<220>
<223> A synthetic construct.

<400> 66
aaccatggct tccaaggtgt acgaccccga gcaacgcaaa 40

<210> 67
<211> 40
<212> DNA
<213> Artificial Sequence

<220>
<223> A synthetic construct.

<400> 67
gctctagaat tactgctcgt tcttcagcac gcgctccacg 40

<210> 68
<211> 31
<212> DNA
<213> Artificial Sequence

<220>
<223> A synthetic construct.

<400> 68
5cgctagccat ggcttcgaaa gtttatgatc c 31

<210> 69
<211> 25
<212> DNA
10<213> Artificial Sequence

<220>
<223> A synthetic construct.

15<400> 69
ggccagtaac tctagaatta ttgtt 25

<210> 70
<211> 1092
20<212> DNA
<213> Artificial Sequence

<220>
<223> A synthetic construct.
25
<400> 70
aagcttgcta gcgccaccat gaagaagccc gagctcaccg ctaccagcgt tgaaaaattt 60
ctcatcgaga agttcgacag tgtgagcgac ctgatgcagt tgtcggaggg cgaagagagc 120
cgagccttca gcttcgatgt cggcggacgc ggctatgtac tgcgggtgaa tagctgcgct 180
30gatggcttct acaaagaccg ctacgtgtac cgccacttcg ccagcgctgc actacccatc 240
cccgaagtgt tggacatcgg cgagttcagc gagagcctga catactgcat cagtagacgc 300
gcccaaggcg ttactctcca agacctcccc gaaacagagc tgcctgctgt gttacagcct 360
gtcgccgaag ctatggatgc tattgccgcc gccgacctca gtcaaaccag cggcttcggc 420
ccattcgggc cccaaggcat cggccagtac acaacctggc gggatttcat ttgcgccatt 480
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gctcaagccc tggacgaact gatgctgtgg gccgaagact gtcccgaggt gcgccacctc 600
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atcgactggt ccgaagctat gttcggggac agtcagtacg aggtggccaa catcttcttc 720
tggcggccct ggctggcttg catggagcag cagactcgct acttcgagcg ccggcatccc 780
40gagctggccg gcagccctcg tctgcgagcc tacatgctgc gcatcggcct ggatcagctc 840
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gccatcgtcc gcagcggggc cggcaccgtc ggtcgcacac aaatcgctcg ccggagcgcc 960
gccgtatgga ccgacggctg cgtcgaggtg ctggccgaca gcggcaaccg ccggcccagt 1020

acacgaccgc gcgctaagga gggtggcgga gggagcggtg gcggaggttc ctacgtatag 1080
tctagactcg ag 1092

<210> 71
<211> 1093
<212> DNA
<213> Artificial Sequence

<220>
<223> A synthetic construct.

<400> 71
aagcttgcta gcgccaccat gaagaagccc gagctcaccg ctaccagcgt tgaaaaattt 60
ctcatcgaga agttcgacag tgtgagcgac ctgatgcagt tgtcggaggg cgaagagagc 120
cgagccttca gcttcgatgt cggcggacgc ggctatgtac tgcgggtgaa tagctgcgct 180
gatggcttct acaaagaccg ctacgtgtac cgccacttcg ccagcgctgc actacccatc 240
cccgaagtgt tggacatcgg cgagttcagc gagagcctga catactgcat cagtagacgc 300
gcccaaggcg ttactctcca agacctcccc gaaacagagc tgcctgctgt gttacagcct 360
gtcgccgaag ctatggatgc tattgccgcc gccgacctca gtcaaaccag cggcttcggc 420
ccattcgggc cccaaggcat cggccagtac acaacctggc gggatttcat ttgcgccatt 480
gctgatcccc atgtctacca ctggcagacc gtgatggacg acaccgtgtc cgccagcgta 540
gctcaagccc tggacgaact gatgctgtgg gccgaagact gtcccgaggt gcgccacctc 600
gtccatgccg acttcggcag caacaacgtc ctgaccgaca acggccgcat caccgccgta 660
atcgactggt ccgaagctat gttcggggac agtcagtacg aggtggccaa catcttcttc 720
tggcggccct ggctggcttg catggagcag cagactcgct acttcgagcg ccggcatccc 780
gagctggccg gcagccctcg tctgcgagcc tacatgctgc gcatcggcct ggatcagctc 840
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gccatcgtcc gcagcggggc cggcaccgtc ggtcgcacac aaatcgctcg ccggagcgca 960
gccgtatgga ccgacggctg cgtcgaggtg ctggccgaca gcggcaaccg ccggcccagt 1020
acacgaccgc gcgctaagga aggcggtgga ggtagtggtg gcggaggtag ctacgtataa 1080
ctctagactc gag 1093

<210> 72
<211> 813
<212> DNA
<213> Artificial Sequence

<220>
<223> A synthetic construct.

<400> 72
gctagcgcca ccatgatcga acaagacggc ctccatgctg gcagtcccgc agcttgggtc 60
gaacgcttgt tcgggtacga ctgggcccag cagaccatcg gatgtagcga tgcggccgtg 120

ttccgtctaa gcgctcaagg ccggcccgtg ctgttcgtga agaccgacct gagcggcgcc 180
ctgaacgagc ttcaagacga ggctgcccgc ctgagctggc tggccaccac cggtgtaccc 240
tgcgccgctg tgttggatgt tgtgaccgaa gccggccggg actggctgct gctgggcgag 300
gtccctggcc aggatctgct gagcagccac cttgcccccg ctgagaaggt ttccatcatg 360
gccgatgcaa tgcggcgcct gcacaccctg gaccccgcta catgcccctt cgaccaccag 420
gctaagcatc ggatcgagcg tgctcggacc cgcatggagg ccggcctggt ggaccaggac 480
gacctggacg aggagcatca gggcctggcc cccgctgaac tgttcgcccg cctgaaagcc 540
cgcatgccgg acggtgagga cctggttgtg acacatggtg atgcctgcct ccctaacatc 600
atggtcgaga atggccgctt ctccggcttc atcgactgcg gtcgcctagg agttgccgac 660
cgctaccagg acatcgccct ggccacccgc gacatcgctg aggagcttgg cggcgagtgg 720
gccgaccgct tcttagtctt gtacggcatc gcagctcccg acagccagcg catcgccttc 780
taccgcctgc tcgacgagtt cttttaatct aga 813

<210> 73
<211> 816
<212> DNA
<213> Artificial Sequence

<220>
<223> A synthetic construct.

<400> 73
gctagcgcca ccatgatcga acaagacggc ctccatgctg gcagtcccgc agcttgggtc 60
gaacgcttgt tcgggtacga ctgggcccag cagaccatcg gatgtagcga tgcggccgtg 120
ttccgtctaa gcgctcaagg ccggcccgtg ctgttcgtga agaccgacct gagcggcgcc 180
ctgaacgagc ttcaagacga ggctgcccgc ctgagctggc tggccaccac cggcgtaccc 240
tgcgccgctg tgttggatgt tgtgaccgaa gccggccggg actggctgct gctgggcgag 300
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gccgatgcaa tgcggcgcct gcacaccctg gaccccgcta cctgcccctt cgaccaccag 420
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atggtcgaga atggccgctt ctccggcttc atcgactgcg gtcgcctagg agttgccgac 660
cgctaccagg acatcgccct ggccacccgc gacatcgctg aggagcttgg cggcgagtgg 720
gccgaccgct tcttagtctt gtacggcatc gcagctcccg acagccagcg catcgccttc 780
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<210> 74
<211> 1252
<212> DNA
<213> Artificial Sequence

<220>
<223> A synthetic construct.

<400> 74
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tacgattcgt gagggcttac catcaggccc cagcgcagca atgatgccgc gagagccgcg 180
ttcaccggcc cccgatttgt cagcaatgaa ccagccagca gggagggccg agcgaagaag 240
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aagaagttcg ccagtgagta gtttccgaag agttgtggcc attgctactg gcatcgtggt 360
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cacatgatca cccatattat gaagaaatgc agtcagctcc ttagggcctc cgatcgttgt 480
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taccgtcatg ccatccgtaa gatgctttтc cgtgaccggc gagtactcaa ccaagtcgtt 600
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cgcgccacat agcagtactt tgaaagtgct catcatcggg aatcgttctt cggggcggaa 720
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<210> 75

<400> 75
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<210> 76
<211> 228
<212> DNA
<213> Artificial Sequence

<220>
<223> A synthetic construct.

<400> 76
actagtcgtc tctcttgaga gaccgcgatc gccaccatga taagtaagta atattaaata 60
agtaaggcct gagtggccct cgagccagcc ttgagttggt tgagtccaag tcacgtctgg 120
aqatctqqta cctacqcqtq aqctctacqt aqctaqcqqc ctcqqcqqcc gaattcttgc 180

gatctaagta agcttggcat tccggtactg ttggtaaagc caccatgg 228

<210> 77
<211> 228
<212> DNA
<213> Artificial Sequence

<220>
<223> A synthetic construct.

<400> 77
actagtacgt ctctcttgag agaccgcgat cgccaccatg ataagtaagt aatattaaat 60
aagtaaggcc tgagtggccc tcgagtccag ccttgagttg gttgagtcca agtcacgtct 120
ggagatctgg taccttacgc gtagagctct acgtagctag cggcctcggc ggccgaattc 180
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<210> 78
<211> 230
<212> DNA
<213> Artificial Sequence

<220>
<223> A synthetic construct.

<400> 78
actagtacgt ctctcttgag agaccgcgat cgcatgccta ggtaggtagt attagagcat 60
aggtagaggc ctaagtggcc ctcgagtcca gccttgagtt ggttgagtcc aagtcacgtc 120
tggagatctg gtaccttacg cgtatgagct ctacgtagct agcggcctcg gcggccgaat 180
tcttgcgatc taagcttggc aatccggtac tgttggtaaa gccaccatgg 230

<210> 79
<211> 234
<212> DNA
<213> Artificial Sequence

<220>
<223> A synthetic construct.

<400> 79
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aagggcttaa aggcctaagt ggccctcgag tccagccttg agttggttga gtccaagtca 120
cgtttggaga tctggtacct tacgcgtatg agctctacgt agctagcggc ctcggcggcc 180
gaattcttgc gatctaagct tggcaatccg gtactgttgg taaagccacc atgg 234

<210> 80
<211> 938
<212> DNA
<213> Artificial Sequence

<220>
<223> A synthetic construct.

<400> 80
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atttccgtgt cgcccttatt ccctttttg cggcattttg ccttcctgtt tttgctcacc 120
cagaaacgct ggtgaaagta aaagatgctg aagatcagtt gggtgcacga gtgggttaca 180
tcgaactgga tctcaacagc ggtaagatcc ttgagagttt tcgccccgaa gaacgttttc 240
caatgatgag cacttttaaa gttctgctat gtggcgcggt attatcccgt attgacgccg 300
ggcaagagca actcggtcgc cgcatacact attctcagaa tgacttggtt gagtactcac 360
cagtcacaga aaagcatctt acggatggca tgacagtaag agaattatgc agtgctgcca 420
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caacaacgtt gcgcaaacta ttaactggcg aactacttac tctagcttcc cggcaacaat 660
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ctggctggtt tattgctgat aaatctggag ccggtgagcg tggctctcgc ggtatcattg 780
cagcactggg gccagatggt aagccctccc gtatcgtagt tatctacacg acggggagtc 840
aggcaactat ggatgaacga aatagacaga tcgctgagat aggtgcctca ctgattaagc 900
attggtaacc actgcagtgg ttttcctttt gcggccgc 938

<210> 81
<211> 938
<212> DNA
<213> Artificial Sequence

<220>
<223> A synthetic construct.

<400> 81
actagtaacc ctgataaatg ctgcaaacat attgaaaaag gaagagtatg agtattcaac 60
atttccgtgt cgcactcatt cccttctttg cggcattttg cttgcctgtt tttgcacacc 120
ccgaaacgct ggtgaaagta aaagatgctg aagatcaact gggtgcacga gtgggctata 180
tcgaactgga tctcaatagc ggtaagatcc ttgagagttt tcgccccgaa gaacgttttc 240
caatgatgag cacttttaaa gttctgctat gtggcgcggt attatcccgt attgacgccg 300
ggcaagagca gctcggtcgc cgcatacact actcacagaa cgacttggtt gagtactcgc 360
cggtcacgga aaagcatctt acggatggca tgacagtaag agaattgtgt agtgctgcca 420
taaccatgag tgataacacc gcggccaact tacttctgac aacgatcgga ggccctaagg 480

agctgaccgc atttttgcac aacatggggg atcatgtaac ccggcttgat cgttgggaac 540
cggagctgaa cgaagccata ccgaacgacg agcgtgacac cacgatgcct gtagcaatgg 600
caacaacgtt gcgcaaacta ctcactggcg aacttctcac tctagcatca cgacagcaac 660
tcatagactg gatggaggcg gataaagttg caggaccact tctgcgctcg gcccttccgg 720
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aggcaactat ggatgaacga aatagacaga tcgctgagat aggtgcctca ctgatcaagc 900
actggtagcc actgcagtgg tttagctttt gcggccgc 938

<210> 82
<211> 938
<212> DNA
<213> Artificial Sequence

<220>
<223> A synthetic construct.

<400> 82
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attttcgtgt cgcactcatt cccttctttg cggcattttg cttgcctgtt tttgcacacc 120
ccgaaacgct ggtgaaagta aaagatgctg aagatcaact gggtgcaaga gtgggctata 180
tcgaactgga tctcaatagc ggcaagatcc ttgagtcttt tcgccccgaa gaacgttttc 240
cgatgatgag cacttttaaa gttctgctat gtggcgcggt gttgtcccgt atagacgccg 300
ggcaagagca gcttggtcgc cgtatacact actcacaaaa cgacttggtt gagtactcgc 360
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ttaccatgag cgacaatacc gcggccaact tacttctgac aacgatcgga ggccctaagg 480
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caacaacgtt gcggaaacta ctcactggcg aacttctcac tctagcatca cgacagcagc 660
tcatagactg gatggaggcg gacaaagtag caggaccact tcttcgctcg gccctccctg 720
ctggctggtt cattgctgat aaatccggtg ccggtgaacg cggctctcgc gggatcattg 780
ctgcgctggg gcctgatggt aagccctcac gaatcgtagt aatctacacg acggggagtc 840
aggccactat ggacgaacga aatagacaga tcgctgagat cggtgcctca ctgatcaagc 900
actggtaacc actgcagtgg tttagcattt gcggccgc 938

<210> 83
<211> 938
<212> DNA
<213> Artificial Sequence

<220>
<223> A synthetic construct.

<400> 83
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ccgaaacgct ggtgaaagta aaagatgctg aagatcaact gggtgcaaga gtgggctata 180
tcgaactgga tctcaatagc ggcaagatcc ttgagtcttt ccgccccgaa gaacgttttc 240
cgatgatgag cactttcaaa gtactgctat gtggcgcggt gttgtcccgt atagacgccg 300
ggcaagagca gcttggtcgc cgtatacact actcacaaaa cgacttggtt gagtactcgc 360
cggtcacgga aaagcatctt acggatggca tgacggtaag agaattgtgt agtgctgcca 420
ttaccatgag cgataatacc gcggccaact tacttctgac aacgatcgga ggccctaagg 480
agctgaccgc atttttgcac aacatgggtg atcatgtgac ccggcttgac cgctgggaac 540
cggagctgaa cgaagccata ccgaacgacg agcgtgacac cacgatgcct gtagcaatgg 600
caacaactct tcggaaacta ctcactggcg aacttctcac tctagcatca cgacagcagc 660
tcatagactg gatggaggcg gacaaagtag caggaccact tcttcgctcg gccctccctg 720
ctggctggtt cattgctgat aaatctggag ccggtgagcg tggctctcgc ggtatcattg 780
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aggccactat ggacgaacga aatagacaga tcgctgagat cggtgcctca ctgatcaagc 900
actggtaacc actgcagtgg tttagcattt gcggccgc 938

<210> 84
<211> 938
<212> DNA
<213> Artificial Sequence

<220>
<223> A synthetic construct.

<400> 84
actagtaacc ctgacaaatg ctgcaaacat attgaaaaag gaagagtatg agcatccaac 60
attttcgtgt cgcactcatt cccttctttg cggcattttg cttgcctgtt tttgcacacc 120
ccgaaacgct ggtgaaagta aaagatgctg aagatcaact gggtgcaaga gtgggctata 180
tcgaactgga tctcaatagc ggcaagatcc ttgagtcttt ccgccccgaa gaacgattcc 240
cgatgatgag cactttcaaa gtactgctat gtggcgcggt gttgtcccgt atagacgccg 300
ggcaagagca gcttggtcgc cgtatacact actcacaaaa cgacttggtt gagtactcgc 360
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cggagctgaa cgaagccata ccgaacgacg agcgtgatac cacgatgcca gtagcaatgg 600
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actggtaacc actgcagtgg tttagcattt gcggccgc 938

<210> 85

<400> 85
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<210> 86

<400> 86
000

<210> 87

<400> 87
000

<210> 88
<211> 1038
<212> DNA
<213> Artificial Sequence

<220>
<223> A synthetic construct.

<400> 88
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gtcggcggac gcggctatgt actgcgggtg aatagctgcg ctgatggctt ctacaaagac 180
cgctacgtgt accgccactt cgccagcgct gcactaccca tccccgaagt gttggacatc 240
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agcaacaacg tcctgaccga caacggccgc atcaccgccg taatcgactg gtccgaagct 660
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gaggtaggtc gagtttaa 1038

<210> 89
<211> 4333
<212> DNA
<213> Artificial Sequence

<220>
<223> A synthetic construct.

<400> 89
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aagaagggcc cagcgccatt ctacccactc gaagacggga ccgccggcga gcagctgcac 180
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(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **WOOD, Keith, V.** [US/US]; 8380 Swan Road, Mt. Horeb, WI 53572 (US). **WOOD, Monika, G.** [US/US]; 8380 Swan Road, Mt. Horeb, WI 53572 (US). **ALMOND, Biran** [US/US]; 5765 Richard Drive, Fitchburg, WI 53719 (US). **PAGUIO, Aileen** [US/US]; 205 Ramsey Court, Madison, WI 53704 (US). **FAN, Frank** [TZ/US]; 2977 Dunmore Street, Madison, WI 53711 (US).

(74) Agents: **STEFFEY, Charles, E.** et al.; Schwegman, Lundberg, Woessner & Kluth, P.A., P.O. Box 2938, Minneapolis, MN 55402 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

(88) Date of publication of the international search report:
26 May 2006

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: SYNTHETIC NUCLEIC ACID MOLECULE AND METHODS OF PREPARATION

(57) Abstract: A method to prepare synthetic nucleic acid molecules having reduced inappropriate or unintended transcriptional characteristics when expressed in a particular host cell.

WO 2006/034061 A3

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2005/033218

A. CLASSIFICATION OF SUBJECT MATTER
C12N15/09 C12N15/31

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, EMBASE, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 01/23541 A (ALEXION PHARMACEUTICALS, INC; FODOR, WILLIAM, L; RAMSOONDAR, JAGDEECE,) 5 April 2001 (2001-04-05) * There is 94,521% identity in 803 nt overlap (total 825 nt) of the sequence shown in Fig. 4 with SEQ ID NO: 4 of the present application *	11,15
A	WO 2004/042010 A (UNIVERSITY OF TENNESSEE RESEARCH FOUNDATION) 21 May 2004 (2004-05-21)	
A	US 5 670 356 A (SHERF ET AL) 23 September 1997 (1997-09-23) cited in the application	

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 March 2006	31.03.06

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Hillenbrand, G

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2002/100076 A1 (GARCON FREDERIC ET AL) 25 July 2002 (2002-07-25) * There is 88,19% identity of SEQ ID NO: 2 in 906 nt overlap (total 5909 nt) with SEQ ID NO: 74 (1252 nt) of the present application - 39,2% identity of SEQ ID NO: 2 to SEQ ID NO: 41 *	47,49,50
X	WO 97/08320 A (MORPHOSYS GESELLSCHAFT FUER PROTEINOPTIMIERUNG MBH; KNAPPIK, ACHIM; PA) 6 March 1997 (1997-03-06) * There is 88,19% identity of the sequence of Fig. 36 in 906 nt overlap (total 1289 nt) with SEQ ID NO: 74 (1252 nt) of the present application - 39,2% identity of SEQ ID NO: 2 to SEQ ID NO: 41 *	47,49,50
X	DATABASE EMBL 1 March 1996 (1996-03-01), GROSKREUTZ ET AL.: "Cloning vector pGL3-Basic, complete sequence" XP002371236 retrieved from EBI Database accession no. U47295 * There is 85,82% identity of U47295 in 3095 nt overlap (total 4818 nt) with SEQ ID NO: 89 (4333 nt) of the present application * abstract	63-67
X	DATABASE EMBL 15 May 2001 (2001-05-15), ZHUANG, Y. ET AL.: "Co-reporter vector phRG-B, complete sequence" XP002371237 retrieved from EBI Database accession no. AF362550 * There is 98,82% identity of AF362550 in 2375 nt overlap (total 4101 nt) with SEQ ID NO: 90 (3522 nt) of the present application * abstract	63-67

INTERNATIONAL SEARCH REPORT

Int[redacted]ional application No.
PCT/US2005/033218

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [ ] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. [X] Claims Nos.: 1-10, 12-14, 16-30, 32-46, 48, 53, 55-62, 68-69
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [X] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

11 and 15 (partially), 47, 49 and 50 (partially), 63-67 (partially)

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[X] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box II.2

Claims Nos.: 1-10, 12-14, 16-30, 32-46, 48, 53, 55-62, 68-69

The present application contains 69 claims, of which 7 claims are independent. They are drafted in such a way that the claims as a whole are not in compliance with the provisions of clarity and conciseness of Article 6 PCT, as they erect a smoke screen in front of the skilled reader when assessing the intended scope of protection. In view of the fact that the starting (parent) nucleic acid sequences are not defined in most claims, it is impossible for the skilled reader to determine the subject-matter for which protection is sought.The non-compliance with the substantive provisions of the PCT is to such an extent, that a meaningful search of the claims identified above was not possible.

The applicant's attention is drawn to the fact that claims relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure. If the application proceeds into the regional phase before the EPO, the applicant is reminded that a search may be carried out during examination before the EPO (see EPO Guideline C-VI, 8.5), should the problems which led to the Article 17(2) declaration be overcome.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

Inventions 1-20 : claims 11 and 15 (partially)

The subject-matter of this group of different inventions comprises an isolated nucleic acid molecule comprising a synthetic nucleotide sequence having a coding region for a selectable polypeptide, wherein the synthetic nucleotide sequence has 90% or less nucleic acid sequence identity to a parent nucleotide encoding a corresponding selectable polypeptide, wherein the nucleotide sequence encodes a selectable polypeptide with at least 85% amino acid sequence identity to the corresponding selectable polypeptide encoded by the the parent nucleotide sequence - wherein the synthetic nucleotide sequence comprises an open reading frame in SEQ ID NO: 4 to SEQ ID NO: 84 as claimed in claims 11 and 15.

Invention 21: claim 31 (partially)

The subject-matter of this invention comprises an isolated nucleic acid sequence encoding a firefly luciferase, wherein the synthetic nucleotide sequence has 80% or less nucleic acid sequence identity to a parent nucleotide having SEQ ID NO: 43 or 85% or less nucleic acid sequence identity to a parent nucleic acid sequence having SEQ ID NO: 14 which encodes a firefly luciferase, wherein the nucleotide sequence encodes a firefly luciferase with at least 85% amino acid sequence identity to the corresponding luciferase encoded by the the parent nucleotide sequence, wherein the synthetic nucleotide sequence comprises an open reading frame in SEQ ID NO: 21-23.

Invention 23: claims 47, 49 and 50 (partially)

A plasmid comprising SEQ ID NO: 74 which comprises an open reading frame with less than 90% nucleic acid sequence identity to 41 which confers resistance to ampicillin.

Inventions 24-46: claims 51-52 (partially)

A polynucleotide which hybridizes under stringent hybridization conditions to SEQ ID NO: 4 to SEQ ID NO: 23 as claimed in claim 51 and encodes a selectable polypeptide or a firefly luciferase.

Invention 47: claim 54

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

An isolated nucleic acid molecule comprising a synthetic nucleotide sequence which does not code for a desirable peptide or polypeptide but includes sequences which inhibit transcription and/or translation wherein the synthetic nucleotide sequence has SEQ ID NO: 49.

Inventions 48-49: claims 63-67 (partially)

A plasmid which includes a sequence including SEQ ID NO : 89 or SEQ ID NO: 90.
The search was limited to matter related to invention 1 and inventions 23, 48 and 49 as requested by the applicant in his letter dated 13.02.2006.

INTERNATIONAL SEARCH REPORT

mation on patent family members

Inter al application No
PCT/US2005/033218

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